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(54) Title: PARAPOXVIRUS VECTORS					
(O) FIRE TAKALOA VIKUS VICTORS					
(57) Abstract					
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#### PARAPOXVIRUS VECTORS

## TECHNICAL FIELD

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This invention relates to parapoxvirus vectors, methods for their construction, and uses thereof.

### **BACKGROUND OF THE INVENTION**

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Poxviruses are large DNA viruses which replicate within the cytoplasm of infected cells. A number of members of the poxvirus family have been used to express foreign genes. These members include vaccinia virus and avipox virus. Such viruses have the potential to deliver vaccine antigens to a variety of animal species. However, the use of modified vaccinia virus and avipox viruses are subject to a number of drawbacks.

Vaccinia virus has a wide host range in mammals. Accordingly, there is a significant risk of cross-species infection and consequent spread of disease from one species to another. This represents a significant disadvantage for any vector being used in the environment.

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A further disadvantage is that vaccinia virus especially, has been shown to cause a febrile response and scarring in humans and occasionally, serious disease in an infected animal.

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Avipoxviruses are more variable in their host range specificity, and while they will not generally propagate in mammals, they will often undergo an abortive infection sufficient to induce an immune response to at least some foreign genes if they are incorporated into the genome of the avipoxvirus and are expressed under control of the appropriate promoter.

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Also the first infection with a vaccinia virus vector will induce an immunity to the vector such that it may limit the potential of a subsequent infection with the vector to deliver a full dose of antigen.

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In the agricultural context, a major limitation to livestock production is the control of parasitic diseases. As drench resistance builds up in farmed animal populations, and consumer resistance to the use of chemical agents in livestock production also increases, there is a need for alternative means of disease control. Use of cheap, safe and effective

vaccines using parapox virus vectors to deliver antigens to the host is one alternative solution which addresses these problems.

The concept of parapox virus vectors and more particularly orf virus vectors is disclosed generally by Robinson, A.J. and Lyttle, D.J. "Parapoxviruses: their biology and potential as recombinant vaccines" in Recombinant Poxviruses, Chapter 9, 306-317 eds M.Binns and G. Smith CRC Press, (1992), Boca Raton. However, there is no teaching in the reference of suitable gene insertion sites or sequences coding therefor which would allow orf virus to be used as a vector.

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It is therefore an object of the present invention to provide a virus vector which goes some way toward overcoming the disadvantages outlined above in relation to existing poxvirus vectors or which at least provides the public with a useful choice.

## 15 SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention provides a parapoxvirus vector comprising a parapox virus containing exogenous DNA.

20 Preferably, the parapox virus is orf virus.

Desirably, the exogenous DNA encodes at least one gene product, and most usefully this product will be an antigen capable of inducing an immune response.

25 In addition, the exogenous DNA preferably further encodes at least one gene product which is a biological effector molecule, most usefully a cytokine which is capable of acting as an immunological adjuvant.

In addition, the exogenous DNA also preferably encodes a peptide moiety expressed as a hybrid or chimeric protein with a native virus protein.

Also within the scope of the invention are fragments or variants of the vector having equivalent immunological activity.

35 It is desirable that the exogenous DNA be incorporated in a non-essential region of the virus genome.

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The exogenous DNA is preferably under the control of a poxvirus promoter, and conveniently an orf virus promoter.

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In a further aspect, the present invention provides a method for the production of parapoxvirus vectors, replicable transfer vectors for use in the method of the invention and hosts transformed with these vectors.

In a further aspect the invention consists in a vaccine which includes a parapoxvirus vector defined above in combination with a pharmaceutically acceptable carrier and optionally or alternatively, an adjuvant therefor.

In a still further aspect the present invention relates to the use of parapoxvirus vectors to prepare heterologous polypeptides in eukaryotic cells comprising infecting cells with the parapoxvirus vector and isolating the heterologous polypeptide once expressed.

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Although the invention is broadly as described above, it will be appreciated by those persons skilled in the art that the invention is not limited to the foregoing but also includes embodiments of which the following gives examples. In particular, certain aspects of the invention will be more clearly understood by having reference to the accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 represents a map of the genomes of the orf virus strains NZ-2, NZ-7 and NZ-10 25 showing cleavage sites for the restriction endonuclease KpnI. The genomes are double stranded DNA molecules and are represented as horizontal lines. The positions of the endonuclease cleavage sites on each genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet.

Figure 2 represents a nucleotide sequence of a region of the Kpnl E fragment of the orf virus strain NZ-2 genome. The sequence underlined with a dashed line contains potential insertion sites. The sequence underlined with colons represents that portion of a vascular endothelial growth factor like gene that contains potential insertion sites.

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Figure 3 represents a nucleotide sequence of a region of the Kpnl D fragment of the orf virus strain NZ-7 genome in Figure 1. The sequences underlined with a dashed line represent sites for the insertion of foreign genes. The sequence underlined with colons represents that portion of a vascular endothelial growth factor-like gene that contains potential insertion sites.

Figure 4 represents a map of the genome of the orf virus strain NZ-2 showing cleavage sites for the restriction endonuclease *Hind*III. The genome is a double stranded DNA molecule and is here represented as a horizontal line. The positions of the endonuclease cleavage sites on the genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet. The region comprising part of fragment F, all of fragments J and I and part of fragment E for which the DNA sequence has been determined is shown. Open reading frames encoding putative genes are shown. The open reading frames encoding the putative genes (H)IIL and (H)I2L contain potential insertion sites. In addition the intergenic regions between rpo132 and (H)I1L, (H)I1L and (H)I2L, (H)I2L and (H)E1L and (H)E2L represent potential insertion sites.

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Figure 5 represents the nucleotide sequence of the open reading frames depicted in Figure 4. The genes (H)I1L, and (H)I2L which contain potential insertion sites are underlined with colons. Potential insertion sites within intergenic regions are underlined with a dotted line. Putative promoter sequences are marked by asterisks.

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Figure 6 represents a map of the genome of the orf virus strain NZ-2 showing cleavage sites for the restriction endonuclease BamHI The genome is a double stranded DNA molecule and is here represented as a horizontal line. The positions of the endonuclease cleavage sites on the genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet. The region comprising fragment BamHI F and part of BamHI C for which the DNA sequence has been determined is shown. Open reading frames encoding DNA topoisomerase (F4R) and the putative genes F1L, F2L, F3R and C1L are shown as unfilled arrows.

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Figure 7 represents a nucleotide sequence of the *BamHI* F fragment and part of the *BamHI* C fragment of the orf virus strain NZ-2 genome shown in Figure 6. The sequences underlined with a dashed line represent potential insertion sites. The putative promoter sequences PF1L, PF2L, PF3R, PF4R and PC1R are marked by asterisks.

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Figure 8 represents a map of the genome of orf virus strain NZ-2 showing cleavage sites for the restriction endonuclease *BamHI*. The genome is a double stranded DNA molecule and is here represented as a horizontal line. The positions of the endonuclease cleavage

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sites on the genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet. The region comprising fragments BamHI H, BamHI E, BamHI G and part of BamHI B for which the DNA sequence has been determined is shown. Open reading frames encoding putative genes are shown as unfilled arrows. The position of a 3.3 kilobase pair deletion encompassing open reading frames E2L, E3L and G1L is shown.

Figure 9 represents a nucleotide sequence of a region of the BamHI E fragment and BamHI G fragment of the orf virus strain NZ-2 genome shown in Figure 8. Potential insertion sites underlined by colons are present in the region which encodes for the putative genes E2L, E3L and G1L. Potential insertion sites within intergenic regions are underlined with a dotted line. Putative promoter sequences are marked by asterisks. The region located between the ITR junction and the marked endpoint of deletion is absent in a variant strain derived from NZ-2.

Figure 10 represents nucleotide sequences from the orf virus genome strain NZ-2 that act as transcriptional promoters. Early and late promoter sequences are indicated. For each sequence the left hand end is the 5' end.

Figure 11 is a diagram representing the steps in the construction of the plasmid pSP-PFlac.

Figure 12 is a diagram representing the steps in the construction of the plasmid pSP-SFPgpt32.

Figure 13 is a diagram representing the steps in the construction of the plasmid pFS-gpt.

Figure 14 is a diagram representing the steps in the construction of the plasmids pVU-DL104 and pVU-DL106.

Figure 15 is a diagram representing the steps in the construction of the plasmids ptov2 and ptov3.

Figure 16 is a diagram representing the steps in the construction of the plasmid ptov6.

Figure 17 is a diagram representing the steps in the construction of the plasmid ptov8.

Figure 18 is a diagram representing the steps in the construction of the plasmids pVU-DL45W and pVU-DL45WI.

Figure 19 is a diagram representing the steps in the construction of the plasmids pVU-5 DL45Wlac and pVU-DL45Wlac.

Figure 20 outlines a strategy for the generation of recombinant orf virus.

Figure 21A provides the nucleic acid sequence for the primers zxs-1, zxs-2, zxs-3 and zxs-10 4 used for the amplification of orf virus sequences used to create the transfer vector pTvec50.

Figure 21B provides the nucleic acid sequence for the modified intergenic region between the RNA polymerase subunit gene, rpo 132, and (H)I1L in pTvec50, showing new created restriction sites for the restriction enzymes Apol, Nsil, Ncol and EcoRl. The priming sites on the original OV sequence for the zxs-3 primer are marked by asterisks, the newly created transcriptional termination signal (TTTTTAT) is shown in bold type.

Figure 22 is a diagram representing the steps in the construction of the plasmids pTvec1 and pTvec-50.

Figure 23 is a diagram representing the steps in the construction of the transfer vectors pTvec50lac-1 and pTvec50lac-2.

In a first aspect the present invention provides a parapoxvirus vector comprising a parapox virus containing exogenous DNA. Preferably, the parapoxvirus is an orf virus. Orf virus has a relatively narrow host range being generally confined to sheep, goats, monkeys and man. The narrow host range avoids the disadvantage associated with the use of vaccinia virus as a vector in the environment. In particular, cross-species infection will be limited.

Most animals and birds would simply undergo an abortive infection of the orf virus, but the orf virus may still be capable of delivering an immunising dose of some antigens.

Accordingly, the narrow host range may allow the use of orf virus in animals normally resistant to infection with orf virus to stimulate an immune response. The orf virus may also be particularly useful in delivering antigens to birds, where the virus does not propagate in avian species.

Orf virus also has the advantage of being less virulent than vaccinia virus in man. Unlike vaccinia virus, orf virus does not cause a febrile response and lesions are shown to heal without scarring. Ideally the orf virus vector will lack its original virulence factor. Orf virus is reviewed in Robinson, A.J. and Balassu, T.C. (1981) Contagious pustular dermatitis (orf). Vet Bull 51 771-761 and Robinson, A.J. and Lyttle, D.J. (1992) "Parapoxviruses: their biology and potential as recombinant vaccines" in Recombinant Poxviruses, Chapter 9, 306-317 eds M.Binns and G. Smith CRC Press, (1992), Boca Raton.

10 The term "containing exogenous DNA" as used herein refers to exogenous DNA which is incorporated into the virus genome.

Preferably, the exogenous DNA in the orf virus vector is a gene encoding a gene product or products. The gene product may be a heterologous peptide or polypeptide but most usefully, the gene product is an antigen or antigens capable of eliciting an immune response in an infected host. Exogenous DNA encoding genes for a combination of antigens is also possible. The antigen(s) may also be treated with suitable inhibitors, modifiers, crosslinkers and/or denaturants to enhance its stability or immunogenicity if required.

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Some examples of foreign genes of medical and veterinary importance which may potentially be incorporated into orf virus include HIV envelope protein, herpes simplex virus glycoprotein, *Taenia ovis* antigens, *Echinococcus granulosus* (hydatids) antigens, *Trichostrongvlus* and antigens of gastrointestinal parasites such as *Haemonchus* and *Ostertagia* or combinations thereof, but are not limited thereto.

Preferred antigens include *Taenia ovis* 45W, 16kd and 18kd antigens as disclosed in WO 94/22913 incorporated herein by reference.

In a further preferred embodiment, the exogenous DNA may further comprise a cytokine gene or genes coding for other biological effector molecules which modify or augment an immune response, in combination with the exogenous antigenic DNA. Preferred cytokine genes include γ interferon and the interleukins comprising IL-1, IL-2, IL-1β, IL-4, IL-5, IL-6, IL-12 and most preferably IL-1, IL-2 and IL-12 either alone or in combination.

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In another embodiment the exogenous DNA may further comprise one or more reporter genes and/or at least one gene coding for a selectable marker.

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Examples of suitable well known reporter genes include Escherichia coli  $\beta$ -galactosidase (lacz), Photinus pyralis firefly luciferase (lux), secreted placental alkaline phosphatase (SEAP) and Aequorea victoria green fluorescent protein (gfp).

5 Selectable marker genes known and suitable for use in the present invention include xanthine-guanine phosphoribosyl transferase gene (xgpt), and neomycin phosphotransferase (aphII)

In a particularly preferred embodiment the exogenous DNA will comprise genes encoding multiple antigens in combination with one or more biological effector DNA molecules to enhance immune response. In practical terms where multiple antigens are coded for they will generally number 20 or less, preferably 10 or less.

Additionally, the DNA preferably encodes a peptide moiety expressed as a hybrid or chimeric protein with a native virus protein.

In this embodiment of the invention the exogenous DNA encodes for a peptide sequence that forms part of a virus protein. The native protein would retain its original properties but would exhibit additional antigenic epitopes, enzymatic properties or receptor-binding functions encoded by the exogenous DNA. Such a chimeric protein could be secreted, or could form part of the virus envelope or could form part of the virus capsid.

Also within the scope of the invention are fragments or variants of a vector of the invention having equivalent immunological activity. Such variants may be produced by the insertion, deletion or substitution of one or more amino acids using techniques known in the art (Sambrook, J. Fritsch, E.F. and Maniatis, T. Molecular Cloning, A Laboratory Manual (Second Edition) Cold Spring Harbour Laboratory Press 1989).

As will be appreciated by the reader, it is also desirable for the foreign gene to be incorporated into a non-essential region of the orf virus genome. In particular, the gene must be inserted into a region where it does not disrupt viral replication.

Surprisingly, the non-essential thymidine kinase gene, which is used as an insertion site in vaccinia virus has not been found in orf virus. It was therefore necessary to identify alternative non-essential sites in orf virus.

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Non-essential sites were identified following restriction enzyme mapping of orf virus DNA. DNA maps for orf virus strains NZ-2, NZ-7 and NZ-10 are shown in accompanying Figure 1.

Potential insertion sites are contained within restriction fragments KpnI E of strain NZ-2, KpnI D of strain NZ-7 and KpnI D of strain NZ-10. Potential insertion sites are located in the restriction fragments BamHI E and BamHI G of strain NZ-2 shown in Figures 8 and 9. Other potential insertion sites have been identified as intergenic regions lying between regions encoding viral genes. Further examples are illustrated in Figures 4 and 5 (restriction fragments HindIII F, J, I and E of strain NZ-2) and in Figures 6 and 7 (restriction fragments BamHI F and C of strain NZ-2). Other insertion sites are also within the scope of the invention, for example, any non-essential gene or intergenic region within the orf virus genomic DNA sequence. Moreover, one or more insertion sites may be selected and used at a time.

There are two currently preferred insertion sites. The first of these sites is the intergenic region between RNA polymerase subunit gene, rpo132 and the open reading frame of the presumptive gene (H) IIL (Figure 4). As shown in Figure 5 this insertion site is 90 nucleotides in length, extending from positions 11 to 96.

- The second of the preferred insertion sites is the *NcoI* site located at the beginning of gene E3L (Figure 8). As shown in Figure 9 this insertion sited is 61 nucleotides in length, extending from positions 2226 to 2286.
- As will also be appreciated, if expression of the foreign gene is to be achieved, it must be under the control of a transcriptional promoter capable of expressing that gene.

A description of poxvirus promoters can be found in Moss, B. (1990). Regulation of vaccinia virus transcription. Annu Rev Biochem. 59, 661-688 incorporated herein by reference. As has been shown, poxvirus RNA polymerase complexes responsible for copying the gene to make a mRNA, will transcribe any gene that is preceded by a poxvirus promoter.

Preferably therefor, the promoter used will be a poxvirus promoter, and particularly a parapoxvirus promoter. The presently preferred promoter is an orf virus promoter. The orf virus promoter may be an early, intermediate or late promoter. Nucleotide sequencing has allowed the identification of a number of orf virus transcriptional promoters including

early, intermediate and late promoters. Orf virus early and late promoters are shown in Figure 10.

One preferred orf virus promoter is the early promoter of the putative gene E1L originally described as ORF-3 by Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. *Virology.* 176, 379-389 and Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional control sequences flank an early gene in the orf parapoxvirus. *Gene.* 97, 207-212.

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Of the late promoters PF1L and PF3R are preferred. Initial studies on the relative strengths and the temporal expression of the promoters indicate that PF3R is an early-late promoter and is therefore the presently preferred promoter for expressing cloned genes encoding antigenic polypeptides. PF1L is a strong late promoter and is the presently preferred promoter for the expression of the  $\beta$ -galactosidase reporter gene. The orientation of the promoter and the gene it controls may be arranged as appropriate. Combinations of promoters may also be employed.

In a further aspect the invention consists in replicable transfer vectors suitable for use in preparing the modified orf virus vector of the invention. Replicable transfer vectors may be constructed according to techniques well known in the art (Sambrook, J, Fritsch, E. F. and Maniatis, T. *Molecular Cloning, A Laboratory Manual* (Second Edition) Cold Spring Harbour Laboratory Press 1989), or may be selected from cloning vectors available in the art.

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The cloning vector may be selected according to the host cell to be used. Useful vectors will generally have the following characteristics:

- (i) the ability to self-replicate;
- (ii) the possession of a single target for any particular restriction endonuclease; and
- (iii) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of vector possessing the aforementioned characteristics are plasmids and bacterial viruses (bacteriophages or phages). Plasmid vectors are preferred for use in the present invention. The plasmid vector will comprise a non-essential region of the orf virus genome. a foreign gene or genes under the control of one or more orf virus

promoters, and a segment of bacterial plasmid DNA. The vector may be a linear DNA molecule but is preferably circular.

In the construction of a modified orf virus it is also an advantage to be able to distinguish the modified virus from the unmodified virus by a convenient and rapid assay. Such assays include measurable colour changes, antibiotic resistance and the like. For rapid assay purposes, the virus vector desirably further includes at least one reporter gene such as lacz, and and/or at least one selectable marker gene such as x-gpt.

In a preferred embodiment, the xanthine-guanine phosphoribosyltransferase gene (x-gpt) and the β-galactosidase gene are inserted into the plasmid vector under the control of suitable orf virus transcriptional promoters. The orientation of the inserted genes may also be important in determining whether recombinants can be recovered from transfections. Figure 14 shows the x-gpt gene in different orientations in pVU-DL101 and pVU-DL102.

In a further aspect, the present invention provides a method for producing a modified orf virus vector. The method comprises transfecting the plasmid cloning vectors defined above into a selected host cell infected with orf virus. Suitable transfection techniques are well known in the art, for example, calcium phosphate-mediated transfection as described by Graham, F. L. and Van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus type 5 DNA. *Virology.* 52, 456-467. Other techniques include electroporation, microinjection, or liposome or spheroplast mediated transfer but are not limited thereto. Preferably, liposome-mediated transfection is used. This method is described by Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*. 84, 7413-7417.

Upon transformation of the selected host with the cloning vector, recombinant or modified orf virus vectors may be produced. The modified virus may be detected by rapid assays as indicated above. For the preferred vectors the presence of the β-galactoside gene is detectable where clones give a blue phenotype on X-gal plates facilitating selection. Once selected, the vectors may be isolated from culture using routine procedures such as freeze-thaw extraction. Purification is effected as necessary using conventional techniques. A strategy for the generation of modified orf virus is shown in Figure 20.

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The transformed host cells also form part of the invention. Many host cells are known in the art including bacterial, insect, plant and animal cells. Preferably, the host cell is a eukaryotic cell. Mammalian host cells are particularly desirable. The preferred host cells of the present invention are primary bovine testis cells or primary ovine testis cells (lamb testis cells).

As will be appreciated, in a further aspect of the invention, the protocol described above may be used to prepare heterologous polypeptides as well as antigens.

In another aspect, the present invention comprises a vaccine preparation comprising the modified orf virus which contains exogenous antigenic DNA, or a fragment or variant thereof having equivalent immunological activity thereto in combination with a pharmaceutically acceptable diluent or carrier and optionally or alternatively an adjuvant. Examples of suitable adjuvants known to those skilled in the art include saponins, Freund's adjuvants, water-in-oil emulsions, glycerol, sorbitol, dextran and many others. Generally, adjuvants will only be used with non-living viral vaccine preparations.

In a further aspect, the present invention comprises a vaccine preparation comprising the modified orf virus which contains exogenous antigenic DNA in combination with exogenous DNA encoding cytokine genes or genes for other biological effector molecules which may modify or augment an existing immune response.

The vaccine may be formulated in any convenient physiologically acceptable form. Vaccine preparation techniques for smallpox are disclosed in Kaplan, *Br. Med Bull.* 25, 131-135 (1969).

Most usefully, the vaccine is formulated for parenteral administration. The term "parenteral" as used herein refers to intravenous, intramuscular, intradermal and subcutaneous injection.

In addition the vaccine may be formulated for oral administration.

Other therapeutic agents may also be used in combination with the vaccine.

Where necessary, the vaccine may be administered several times over a defined period to maximise the antibody response to the foreign antigen.

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Other methods for inserting foreign genes into orf virus are also contemplated. Potentially, a restriction endonuclease that cuts orf virus DNA once may be used. The cleaved site may be removed following in vitro mutagenesis followed by joining by ligation. If the site is in an essential gene the mutagenesis may be arranged such that the gene function is not affected. This is possible by substituting a base in a codon that lies wholly or partly in the restriction endonuclease cleavage site with another base that allows the new codon to code for the same amino acid but for that substitution to remove the cleavage site for that particular restriction endonuclease. The cleavage site could then be created within any non-essential gene by mutagenesis. This cleavage site then acts as a site for the insertion of foreign genes. The insertion of foreign genes may be done outside the cell by removing the phosphate from the cleaved ends of the DNA to prevent recreation of uninterrupted orf virus DNA, joining a foreign gene which has phosphorylated ends into the orf virus DNA in a ligation reaction and then transfecting the resulting ligation mixture into cells permissive for orf virus. To recover the virus the 15 cell is infected with a poxvirus that was non-permissive for those cells, for instance fowlpox virus and primary bovine testis cells.

Non-limiting examples will now be provided.

#### Example 1 - Selection of a Suitable Cell Culture System 20

The source of cells for culture in the methods described in this application was calves of between one day and three months of age. The testicles were removed from the scrotum of the animal without anaesthetic by a veterinarian skilled in this procedure. The testicles 25 were removed with the tunica parietalis intact to keep the culture cells sterile. The tissue was transported on ice to the laboratory, and the testicular tissue removed from the testis, dispersed into single cells and small aggregates of cells and incubated in suitable culture vessels in culture medium by sterile procedures familiar to those skilled in the art.

## Example 2 - Identification of Insertion Sites

The DNAs of various orf virus isolates have been physically mapped using restriction endonucleases. Such mapping has revealed that there are many different strains of the virus that can be distinguished by the size and order of the restriction endonucleasegenerated fragments although strains may not necessarily differ in their phenotype. From this data it was noted that there was a difference in size between two strains in a restriction endonuclease KpnI fragment mapping to the right end of the genome (Robinson A.J., Barns, G., Fraser, K. Carpenter, E. and Mercer, A.A. (1987). Conservation and variation in orf virus genomes. Virology. 157, 13-23). These two strains were designated NZ-2 and NZ-7 and the fragments KpnI E and KpnI D respectively. NZ-7 contained the larger of the two fragments. The difference in size was about 1 kilobase pair. Another strain designated NZ-10 was seen to have a fragment, fragment KpnI D intermediate in size between the corresponding fragments in NZ-2 and NZ-7 but located in the same relative position in the genome (see Fig.1). This variability suggested that all or part of the region was non-essential and that within this fragment, a site in which to insert foreign DNA might be found. The regions described have subsequently been sequenced and potential insertion sites identified (Fig. 2 and Fig. 3).

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Another potential insertion site was identified when DNA/DNA hybridization between strains, for example between NZ-2 and NZ-7, detected a region of non-homology extending over 2.75 kilobase pairs and this was mapped to a region about 30 kilobase pairs from the right end of the genome (Robinson A. J., Barns, G., Fraser, K, Carpenter, E. and Mercer, A. A. (1987). Conservation and variation in orf virus genomes. *Virology*. 157, 13-23 and Naase, M., Nicholson, B. H., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). An orf virus sequence showing homology to the fusion protein gene of vaccinia virus. *J. Gen Virol.* 72, 1177-1181) (Fig. 4). This region was then completely sequenced and two genes, HI1L and HI2L identified, each of which contains potential insertion sites (Fig. 5).

A third potential insertion site was located in the centre of the genome where a size difference of 100 base pairs was seen between the *Bam*HI G fragment in a strain designated NZ-41 and equivalent region in the other strains examined (Robinson, A. J., Barns, G., Fraser, K., Carpenter, E. and Mercer, A. A. (1987). Conservation and variation in orf virus genomes. *Virology*. 157, 13-23). The nucleotide sequence of the equivalent region in the genome of strain NZ-2, the *Bam*HI F fragment, has been determined and two potential insertion sites identified (Fig. 6 and Fig. 7).

Fourthly, a spontaneous re-arrangement of the orf virus genome of strain NZ-2 was detected following serial propagation of the virus in cell culture. This re-arrangement resulted in the addition of 16 kilobase pairs of right-end DNA sequences to the left end and the deletion of 3.3 kilobase pairs of DNA from the left end. Genomic analysis of a transposition-deletion variant of orf virus reveals a 3.3 kbp region of non-essential DNA (Fleming, S. B., Lyttle, D. J., Sullivan, J. T., Mercer, A. A. and Robinson, A. J. (1995). J Gen Virol., 76, 2969-2978). The order of nucleotides making up the region of the genome that can tolerate a deletion has been deduced by the method of Sanger and three genes contained therein identified. These genes correspond to E2L, formerly ORF-1

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(Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. Virology. 176, 379-389), E3L formerly ORF-PP (Mercer, A. A., Fraser, K., Stockwell, P. A. and Robinson, A. J. (1989). A homologue of retroviral pseudoproteases in the parapoxvirus, 5 orf virus. Virology 172, 665-668) and G1L (Sullivan, J. T., Fraser, K., Fleming, S. B., Robinson, A. J. and Mercer, A. A. (1995). Sequence and transcriptional analysis of an orf virus gene encoding ankyrin-like repeat sequences. Virus Genes, 9, 277-282). This region (Fig. 8) is another potential site for gene insertion (see Fig. 9).

### 10 Example 3 - Identification of Orf Virus Promoters

Determining the nucleotide sequence of selected regions of the orf virus genome has allowed the identification of a number of orf virus transcriptional promoters, in the first instance by virtue of their similarity to other poxvirus transcriptional promoters, and later 15 by functional assays.

Orf virus early and late promoters are shown in Figure 10. The early promoter E1L (ORF-3) was shown to make mRNA early in the cell cycle (Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional 20 control sequences flank an early gene in the orf parapoxvirus. Gene. 97, 207-212) and the late promoter F1L was deduced to be a late promoter by virtue of its similarity to a vaccinia virus late promoter. The orf virus late promoter is functional in a transient assay. Such assays have been described for instance by (Cochran, M. A., Mackett, M. and Moss. B. (1985). Eukaryotic transient expression system dependent on transcription factors and 25 regulatory DNA sequences of vaccinia virus. Proc Natl Acad Sci USA. 82, 19-23). A third promoter F3R, identified as an early-late promoter, is also shown to be functional in a transient assay. The construction of a plasmid pSP-PFlac containing the orf virus late promoter, F1L, and the E. coli gene for B-galactosidase (lacz) such that the Bgalactosidase gene is under the control of the orf virus late promoter is described in 30 Example 6 and illustrated in Figure 11.

## (A) Assessment of Promoter Activity in Transient Assay

To show that the promoter is active in a transient assay, a confluent monolayer of bovine testis cells, in a plastic flask of 25 cm2 surface area for the adherence of the cells and 35 suitable for cell culture work, was infected with orf virus at a multiplicity of infection of approximately 10 plaque forming units per cell. Two hours after infection, the plasmid containing the lacz gene linked to the promoter under investigation was introduced into

orf virus infected bovine testis cells using the liposome mediated transfer technique as described by (Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*. 84, 7413-7417) and as set forth in Example B. Forty eight hours after infection, 35 µ1 of a solution of 5-bromo-4-chloro-3-indolyl-\(\beta\)-D galactosidase (X-gal) at a concentration of 2% w/v in water was added to 1 ml of 1% agarose in cell culture medium which was overlayed onto the cells after the removal of the liquid medium and allowed to form a gel at room temperature (in the range of 15\*-25\* C). Over the succeeding 24 hours the development of a blue coloration in the cells and in the gel above the affected cells was looked for. The development of a blue coloration greater than that seen in cells treated similarly, but with a plasmid containing the \(\beta\)-galactosidase gene not under control of a transcriptional promoter, indicated that the promoter being tested was active.

In a further aspect of investigating promoter function a quantitative assay for βgalactosidase activity in transiently-infected bovine testis cells is performed. Cells are grown as confluent monolayers in multiwell plastic tissue culture trays containing 24 wells 1.5 cm in diameter. Individual wells are infected with orf virus at a moi of 10 and two hours after infection the plasmid construct containing the promoter linked to the  $\beta$ galactosidase gene is introduced into the infected cells using the liposome mediated transfection technique described above. Cells are harvested by scraping into a 1 ml volume of phosphate-buffered saline (PBS), collected by centrifugation, washed with PBS and resuspended in a 200µl volume of PBS. Cells are disrupted by three cycles of freezing and thawing, centrifuged, and the supernatant retained for the enzyme assay. The assay for  $\beta$ -galactosidase is conveniently performed in 96-well microtitre trays. The reaction mixture of 0.1 ml contains 100mM Na-phosphate, pH 7.3, 1mM MgCl2, 50mM β-mercaptoethanol, O-nitrophenyl-β-D-galactoside (ONPG) at a final concentration of 1.3mg/ml and a 10-20µl aliquot of the cell lysate. The reaction mix is incubated at 370 C for 1 hour and the reaction is terminated by the addition equal volume of 1M NaCO3. The absorbance of each well is measured at 420 nm using a microtitre plate reader. The absorbance value is proportional to the amount of B-galactosidase activity present in the original extract and this enables the time course of expression and the relative strength of each promoter construct to be determined.

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# Example 4 - Construction of a Vector Plasmid Suitable for the Insertion of Foreign Genes into the Orf Virus Genome

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The choice of non-essential DNA was the region discovered to be deleted in a re-arranged mutant of orf virus and the relevant sequence of nucleotides in this region can be found in Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. Virology. 176, 379-389 and in Sullivan, J. T., Fraser, K. M., Fleming, S. B., Robinson, A. J. and Mercer, A. A. (1995). Sequence and transcriptional analysis of an orf virus gene encoding ankyrin-like repeat sequences. Virus Genes 9, 277-282 and is shown in Figure 8. The orf virus promoters used were an early promoter, E1L, described in Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. Virology. 176, 379-389 and Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional control sequences flank an early gene in the orf virus. Gene. 97, 207-212 and a late promoter F1L (Fleming, S. B., Blok, J., Fraser, K. M., Mercer, A. A. and Robinson, A. A. (1993). Conservation of gene structure and arrangement between vaccinia virus and orf virus. Virology. 195, 175-184) as shown in Figure 10. The foreign genes chosen to demonstrate the process of creating a mutated orf virus were the E. coli B-galactosidase gene, which has the advantage that when expressed the protein product can be detected by a colour reaction (Miller, J. H. (1972). "Experiments in Molecular Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Moss, B. (1990). "Poxviridae and their Replication" in Virology, Fields et al., eds, 2nd ed. Raven Press, New York, 2079-2111), and the E. coli guanyl phoshoribosyl transferase (x-gpt) gene which when expressed can be used to select mutants from unmutated virus (Mulligan, R. C. and Berg, P. (1980). Expression of a bacterial gene in mammalian cells. Science. 209, 1422-1427). The following is a description of the construction of the vector plasmid. Figures 11 -13 outline the construction in diagrammatic form.

## (A) Cloning an Orf Virus Late Promoter in Front of the E. coli LacZ Gene

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In the construction of a mutant orf virus it is an advantage to be able to distinguish mutant virus from unmutated virus by a convenient and rapid assay. Such an assay is provided by inserting the *E. coli* gene for the \(\beta\)-galactosidase enzyme under control of an orf virus transcriptional promoter into the vector plasmid. The late orf virus promoter was identified by determining the nucleotide sequence of a fragment of orf virus DNA designated \(\beta\) amHI F (Fleming. S. B., Blok, J., Fraser, K. M., Mercer, A. A. and Robinson.

A. A. (1993). Conservation of gene structure and arrangement between vaccinia virus and orf virus. Virology. 195, 175-184). The sequence of the promoter F1L used in this construction is shown in Fig. 10. A sufficient quantity of the late promoter for the construction can be obtained from the plasmid designated pVU-6 which has been described (Mercer, A. A., Fraser, K., Barns, G. and Robinson, A. J. (1987). The structure and cloning of orf virus DNA. Virology. 157, 1-12). A total of 2.62 kb of DNA is deleted from the BamHI F fragment of orf NZ-2 by digesting the plasmid pVU-6, which contains the BamHI F fragment of orf NZ-2 cloned into the plasmid pUC-8 (Viera, J. and Messing, J. (1982). The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene. 19, 259-268) with Aval. This enzyme cleaves the Smal site of the pUC-8 polylinker and six internal Aval sites in BamHl E. The Aval sites remaining on the vector fragment are end-filled with Klenow DNA polymerase, and religated to give the plasmid pVU-Av6. The plasmid pVU-Av6 is cut with BamHI and EcoRI releasing a 725 bp fragment containing the orf virus late promoter. This fragment is cloned into pMLB 1034 (Weinstock, G. M., Berman, M. L. and Silhavy, T. J. (1983). "Chimeric genetics with B-galactosidase in gene amplification and analysis." in Expression of Cloned Genes in Procaryotic and Eucaryotic Cells, Papas et al., eds. Elsevier, New York, 27-64) which contains a "headless" lacz gene. This cloning places the orf virus late promoter in front of lacz and supplies it with an ATG initiation codon allowing the synthesis of B-galactosidase. The colonies that result from this cloning step give a blue phenotype on X-gal plates facilitating the selection of the required clone. A unique Ball site downstream from the lacz insert of pMLB-1034 is converted to an EcoRI site by the following cloning steps. The Tn5 aminoglycoside 3' phosphotransferase gene is released from the plasmid pNEO (Beck, E., Ludwig, A., Aurswald, E. A., Reiss, B. and Schaller, H. (1982). Nucleotide sequence and exact location of the neomycin phosphotransferase from transposon Tn5. Gene. 19, 327-336) with EcoRI and BamHI. The restriction sites are end-filled with Klenow DNA polymerase and the fragment ligated into plasmid pMLB-PF which had been cut with Ball. Recombinants are selected by plating on kanamycin medium. This creates an EcoRI or BamHI site at the position of the original BalI site depending on the orientation of the cloned aminoglycoside 3'-phosphotransferase II (aphII) gene. Ball often cuts DNA inefficiently, but the method allows for the selection of the plasmids which have been cut by Ball and have received the insert, consequently becoming modified in the desired manner. The plasmid pMLB-PFneo is cut with EcoRI and a 4059 bp EcoRI fragment containing the PF-lacZ fusion is cloned into pSP-70 (Melton, D. A., P.A., R., Rebagliati, M. R., Maniatis, T., Zinn, R. and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing

bacteriophage SP6 promoter. *Nucleic Acids Res.* 12, 7035-7056) at the *EcoRI* site to give the plasmid designated pSP-PFlac shown in the diagram Fig. 11.

## (B) Cloning of an Orf Virus Early Promoter in Front of the E. coli X-GPT Gene

In the construction of the mutated orf virus, a means of selecting mutants from nonmutants, from a mixture of both, is required. A method that has been used by others is to utilise the guanyl phosphoribosyl transferase gene of E. coli. Resistance is conferred to a metabolic inhibitor, mycophenolic acid, when the gene is expressed in a eukaryotic cell. A method for incorporating this gene into a vector plasmid under the control of an early promoter is described by Falkner, F. G. and Moss, B. (1988). Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J Virol. 62, 1849-1854 and Boyle, D. B. and Coupar, B. E. (1988). Construction of recombinant fowlpox viruses as vectors for poultry vaccines. Virus Res. 10, 343-356. A plasmid designated pVU-5 is used to provide an early orf virus promoter. The plasmid pVU-5 contains the orf virus NZ-2 BamHI E fragment cloned into pUC-8 and the construction of this plasmid is described in Mercer, A. A., Fraser, K., Barns, G. and Robinson, A. J. (1987). The structure and cloning of orf virus DNA. Virology. 157, 1-12. An early promoter E1L has been described for the putative gene originally designated ORF-3 in pVU-5 by Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. Virology, 176, 379-389 and by Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional control sequences flank an early gene in the orf parapoxvirus. Gene. 97, 207-212; and it is this early promoter that is used in the method described in this application to construct a mutant orf virus. A 503 bp Alul A+T-rich fragment shown in the Fig. 12 is cleaved from pVU-5 and cloned into the HincII site of the multifunctional plasmid vector pTZ18R described in Mead, D. A., Szczesna-Skorupa, E. and Kemper, B. (1986). Single-stranded DNA "blue" T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein 30 Eng. 1, 67-74 giving pSFAlu-6. Plasmid pSFAlu-6 is cut with Ddel and the fragments end-filled with Klenow DNA polymerase. The fragments are recut with HindIII and a 467 bp HindIII- DdeI fragment ligated into pSP-70 which is prepared by cutting with BgIII, end-filling and recutting with HindIII. The resulting plasmid pSP-SFP retains the Bg/II site which is reformed during the cloning step. The plasmid pSV-gpt2, containing the E. coli x-gpt gene, (Mulligan, R. C. and Berg, P. (1981). Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyl transferase. Proc Natl Acad Sci USA. 78, 2072-2076) is cut with BamHI and Bg/II. This releases the x-gpt gene as a 1788 bp fragment which is then cloned into the Bg/II site of pSP-SFP.

fusing the orf virus fragment to the x-gpt gene giving pSP-SFPgpt32. The plasmid pVU-5 is then cut with Smal and Sphl. A 150 bp Smal-Sphl fragment containing the early promoter E1L, the sequence of which is shown in Fig. 10, is cloned into pTZ18R between the Smal and SphI sites giving the plasmid pFS-1. The plasmid pFS-1 is cut with 5 SphI and incubated with T4 DNA polymerase. The aphII gene is released from the plasmid pNEO with EcoRI and BamHI. The EcoRI and BamHI sites are end-filled with Klenow DNA polymerase and the fragment ligated into pFS-1. The resulting plasmid pFS-neo3 contains the aphII gene flanked by an EcoRI site and a BamHI site which lies between it and the early orf virus promoter. A result of these manipulations is that the SphI site distal to the early promoter is converted to a BamHI site. The aphII gene and the early promoter lie in a "head-to-head" orientation and may be removed by digestion with EcoRI. Next, the plasmid pSP-sSFPgpt32 is cut with Pvull. The aphIl-early promoter construct was cut out of pFSneo3 with EcoRI, end-filled with Klenow DNA polymerase, and ligated into the PvuII site. A plasmid termed FSneo-SFPgpt which contains the early promoter running in the same direction as the 503 bp Alul fragment is selected. The plasmid FSneo-SFPgpt is cut with BamHI and BgIII. This step removes the sequence between nucleotides a and b (Fig. 13) together with the aphll gene as a BamHI-Bg/II fragment. The vector fragment is subjected to electrophoresis in an agarose gel and then purified using the powdered glass milk method described by (Vogelstein, B. and 20 Gillespie, D. (1979). Preparation and analytical purification of DNA from agarose. Proc Natl Acad Sci USA. 76, 615-619) and the free Baml and BglII termini ligated together fusing the early promoter to the x-gpt gene. The net result of the manipulations described in steps 4, 5, 6, and 7 (Fig. 13) was to replace the sequence between nucleotides a and b in pSP-SFPgpt32 with the FS promoter forming pFS-gpt.

Example 5 - Identification of a Non-essential Region of the Orf Virus Genome and Insertion of this Site into a Plasmid

A gene coding, potentially, for a peptide of 159 amino acids was found from the sequencing of the 4.47 kb BamHI E fragment which spans the ITR junction of the orf virus genome. This was termed E3L (ORF-PP) and shows homology to an open reading frame in retroviruses (Mercer, A. A., Fraser, K. M., Stockwell, P. A. and Robinson, A. J. (1989). A homologue of retroviral pseudoproteases in the parapoxvirus, orf virus. Virology. 172, 665-668) and to E. coli dUTPase (McGeoch, D. J. (1990). Protein sequence comparisons show that the 'pseudoproteases' encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family. Nucleic Acids Res. 18, 4105-4110). A spontaneous mutant of orf virus isolated in the laboratory was found not to contain the E3L gene due to a complex rearrangement involving the deletion of part of

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the BamHI E fragment and duplication of DNA segments from the opposite end of the genome at that locus. The E3L gene is therefore non-essential and was chosen as a target for the insertion of foreign DNA and to demonstrate that orf virus could tolerate the insertion of a foreign gene. A 2587 bp Smal-BamHI fragment (Fig. 14) containing the unique region of NZ-2 BamHI E is cut out of pVU-5 and cloned into pSP-70 cut with PvuII and Bg/II. The resulting plasmid, pVU-DL100 contains a unique NcoI site that lies between the coding sequence of the E3L gene and its promoter.

# Example 6 - Insertion of the E. coli X-GPT and Lac Z Gene Constructs into pVU-DL100 to Create a Vector Plasmid

Plasmid pVU-DL100 is cut with Ncol and end-filled with Klenow polymerase. The E3L-gpt construct is cut from pFSP-gpt with EcoRI and DraI, end-filled with Klenow polymerase and ligated into pVU-DL100 at the Ncol site. Ligation of the end-filled EcoRI site of the insert to the end-filled Ncol site on the plasmid creates an EcoRI site upstream of the early promoter. The insert is recovered in two orientations, pVU-DL101 with the x-gpt gene running in the opposite direction to the pseudoprotease gene and pVU-DL102 with the x-gpt gene running in the same direction as the pseudoprotease gene. The F1L-lac construct is cut out of pSP-PFlac with EcoRI and cloned into the EcoRI sites of both pVU-DL101 and pVU-DL102. Four plasmids with different orientations of the inserted fragments are recovered from the cloning but only two, pVU-DL104 derived from pVU-DL101, and pVU-DL106 derived from pVU-DL102 which contain the E3L-gpt and F1L-lac in the "back-to-back" orientation are used for transfection experiments.

## 25 Example 7- Constructing a Chimeric Gene Expressing the T. ovis 45W antigen.

A 64 bp fragment of the VEGF like-gene from orf virus NZ-7 (Lyttle, D. J., Fraser, K. M., Fleming, S. B., Mercer, A. A. and Robinson, A. J. (1993) Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol. 68, 84-92) containing five 3' prime terminal codons, the translational termination codon TAA, and a poxvirus transcriptional terminator sequence 5TNT, was amplified using a pair of oligonucleotide primers designed to provide a Bg/II and a Ncol restriction site flanking the amplified sequence. This fragment was digested with Bg/II and Ncol and ligated into the vector pSL301 (Brosius, J. (1989) Superlinkers in cloning and expression vectors. DNA 8, 759-777) cut with BgIII and Ncol to form the plasmid ptov1. A DNA fragment containing the aphII gene and the F1L and F3R promoters of orf virus was amplified by PCR using specific primers which introduced a MluI site at one end and a NsiI and EcoRI site at the other end. One portion of the amplified product was digested with MluI and

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EcoRI and ligated into ptov1 cut with MluI and EcoRI to create the plasmid ptov2. A second portion was digested with MluI and NsiI and ligated into ptov1 to form the plasmid ptov3. The steps showing this construction are illustrated in Figure 15.

The aphII gene was removed from the plasmid ptov2 by digesting with the restriction enzymes BamHI and Bg/II, purifying the vector fragment and re-ligating the free ends to form the plasmid ptov5. The DNA sequence encoding the Taenia ovis 45W antigen fragment was removed from the plasmid pGEX 45W (Johnson, K. S., Harrison, G. B. L., Lightowlers, M. W., O'Hoy, K. L., Cougle, W. G., Dempster, R. P., Lawrence, S. B., Vinton, J. G., Heath, D. D., and Rickard, M. D. (1989). Vaccination against ovine 10 cysticercosis using a defined recombinant antigen. (Nature 338, 585-587) by digesting with the restriction enzymes EcoRI and Bam HI and ligating it into ptov5 cut with BamHI and EcoRI to form ptov6. This placed the DNA sequence encoding the 45W antigen fragment under the control of the orf virus PF3R promoter and supplied it with translational and transcriptional termination sequences. These steps are illustrated in Figure 16.

A 73 bp fragment from the 5' portion of the VEGF-like gene from orf virus NZ-7 encoding the presumptive secretory leader sequence was amplified with specific primers which introduced a new initiation codon, a PstI and an EcoRI restriction site into the amplified DNA fragment. The amplified fragment was digested with PstI and EcoRI and cloned into ptov3 cut with NsiI and EcoRI to create the plasmid ptov4. The plasmid ptov4 was digested with BamHI to remove the aphII gene, purified by agarose gel electrophoresis and religated to form the ptov7. The DNA sequence encoding the 45W 25 antigen fragment was removed from the plasmid pGEX 45W by digesting with the restriction enzymes EcoRI and Bam HI and ligating it into ptov7 cut with BamHI and EcoRI to form ptov8. This placed the 45W antigen fragment under the control of the orf virus PF3R promoter and supplied a 5' protein secretory leader sequence in addition to the 3' translational and transcriptional terminators present in ptov6. These steps are illustrated in Figure 17.

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The plasmid pVU-DL101 was cut with EcoRI and an oligonucleotide linker containing a BamHI and a Ncol restriction site was ligated in to form the plasmid pVU DL101L4. This plasmid was then digested with BamHI and Ncol to allow the insertion of both versions of the chimeric 45W gene from ptov6 and from ptov8. The resulting plasmids were designated pVU-dl45W (from ptov6) and pVU-dl45Wl (from ptov8). These steps are illustrated in Figure 18.

A promoterless *lacz* gene was cleaved out of the plasmid pVUsp-PF2lac, a derivative of pSP PFlac illustrated in Fig. 11 by digestion with BamH1 and BglII. In this latter version of the plasmid, the F1L promoter fragment has been truncated to 100 base pairs and a *Bgl*II restriction site introduced distal to the *lacz gene*. The *lacz* fragment was gel purified and ligated into both pVU-DL45W and pVU-Dl45Wl at a unique *Bam*H1 site. This placed the *lacz* gene under the control of the F1L promoter and completed the construction of the transfer vectors for introducing the *T. ovis* 45W gene into the orf virus genome. These steps are illustrated in Figure 19.

10 The same oligonucleotide linker containing the BamHI and a NcoI restriction sites was ligated into the plasmid pVU-DL102. This plasmid contains the x-gpt gene cloned in the opposite orientation to that in pVU-DL101 (Fig 14). Cloning steps parallel to those described for pVU-DL101 were subsequently performed and the transfer vectors which were generated were designated pVU-DL45W6lac and pVU-DL45W8lac. These contained the same sequences as pVU-DL45Wlac and pVU-DL45Wlac respectively, but differed in that the entire inserted region was in the opposite orientation to that illustrated for these plasmids in Fig. 19.

## **Example 8 - Transfection Protocol**

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Primary bovine testis (BT) cells were grown in monolayer cultures in Eagle's Minimal Essential Medium (MEM; Sigma Cat. No. M0643) supplemented with lactalbumin hydrolysate (5 g/L) and 5% foetal calf serum. Medium for selecting orf virus transformants expressing x-gpt contain mycophenolic acid, 25 μg/ml, xanthine, 250 μg/ml, hypoxanthine, 15 μg/ml, aminopterin, 1 μg/ml, thymidine, 5 μg/ml and 2% foetal calf serum. Lactalbumin hydrolysate was omitted from the selective medium and replaced with additional non-essential amino acids (MEM non-essential amino acid mixture, Sigma Cat. No. M2025).

BT cells were grown as monolayers in a suitable cell culture vessel. Twenty-four hours prior to infection, the cell growth medium was replaced with the selective medium containing mycophenolic acid. The cells were infected with orf virus, strain NZ-2, (moi 0.05 - 0.1) and the virus allowed to adsorb for 1 hour. Cell monolayers were washed 2 times with opti-MEM serum-free medium, (Life Technologies Inc, Gaithersburg, MD U.S.A.) to remove residual foetal calf serum, and drained. A 1.0 ml volume of opti-MEM containing 10. µl Lipofectin reagent (Life Technologies Inc, Gaithersburg, MD, U.S.A.) and approximately 2.0 µg plasmid DNA diluted according to the suppliers instructions was added to each flask and incubated overnight. Following this overnight adsorption

step, 5.0 ml of selective medium containing 2% foetal calf serum was added and the incubation continued until cytopathic effect (CPE) was observed approximately 3 - 5 days post-infection.

Cell monolayers were scraped from the flask, deposited in the bottom of a centrifuge tube by low speed centrifugation, washed with phosphate buffered saline (PBS) and resuspended in PBS. A suitable tissue culture vessel was seeded with BT cells to produce a confluent monolayer. Routinely, 60mm diameter polystyrene dishes were used, seeded with 1.5 x 106 cells per dish and incubated in a CO<sub>2</sub> atmosphere to maintain a pH of around 7.2. The culture medium was removed and 0.5 ml of an appropriate dilution of orf virus in PBS was added and incubated for one hour at 37°C. Dishes were tipped at 15 min intervals to ensure an even distribution of fluid. At the end of this time the inoculum was removed and growth medium containing 1% agarose added. After five days, the time when plaques usually become visible, X-gal was added to the dish in a 1% agarose overlay and incubated a further 12 hours for colour development to occur. Single plaques are picked, resuspended in PBS and inoculated into a partially drained cell culture vessel which had been seeded with 2 x 105 cells and grown to confluence as described. One ml of medium was added to each well and incubation at 37°C continued until a complete cytopathic effect was observed. The cell culture vessels were placed at -20°C until the contents were frozen after which time they were thawed. The cell lysates were used as 20 a source of virus, for further plaque purification, and of viral DNA for hybridisation. Viral DNA was prepared from cytoplasmic extracts of BT cells by the method of Moyer, R. W. and Graves, R. L. (1981). The mechanism of cytoplasmic orthopoxvirus DNA replication. Cell. 27, 391-401. The isolated DNA was digested with restriction enzymes to confirm the insertion of the foreign genes. Frequently, the first plaque purification step 25 fails to remove all the wild type virus and a series of plaque purification steps may be performed in order to obtain a pure culture of mutated virus. Bulk cultures of virus are grown in 150 cm2 tissue culture flasks and the virus purified by the method described in Robinson, A. J., Ellis, G. and Balassu, T. (1982). The genome of orf virus: restriction endonuclease analysis of viral DNA isolated from lesions of orf in sheep. Arch Virol. 71, 43-55. DNA is extracted from the purified virions by the method described in Balassu, T. C. and Robinson, A. J. (1987). Orf virus replication in bovine testis cells: kinetics of viral DNA, polypeptide, and infectious virus production and analysis of virion polypeptides. Arch Virol. 97, 267-281.

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## Example 9 - Assessment of Orf Virus Modification

In order to determine whether or not the viruses recovered from the transfections and plaque purifications were modified to carry the inserted genes, DNA was prepared from 5 infected cells and tested by hybridisation by methods well known to those skilled in the art, for example, Merchlinsky, M. and Moss, B. (1989). Resolution of vaccinia virus DNA concatemer junctions requires late-gene expression. J Virol. 63, 1595-1603. In the preparation of mutated orf virus DNA for these tests, a 100 µl aliquot of orf virus-infected BT cells in PBS was centrifuged for 30 min at approximately 12,000g. The cell pellet was resuspended in 50 µl 0.15M NaCl, 20mM Tris, 10 mM EDTA, pH 8.0. A 250 µl volume of 20mM Tris, 10mM EDTA, 0.75% SDS containing a protease at an appropriate concentration (e.g. Proteinase K at 0.5 mg/ml) was added to each sample and incubated The samples were extracted with an equal volume of at 370 C for 3 hours. phenol:chloroform (1:1) before precipitation with ethanol. Following centrifugation the 15 ethanol-precipitated DNA was redissolved in 50 µl TE. The material harvested from the various passages was subjected to the hybridization procedure with a specific x-gpt probe. A positive result can be obtained with pVU-DL106 for the transfection two hours postinfection as early as passage one. An alternative procedure that was used to detect heterologous DNA markers in recombinant virus was to amplify DNA sequences by the 20 polymerase chain reaction using primers specifically designed to amplify the foreign DNA sequences. Other transfections may require further passages for the detection of recombinant viruses. Transfections performed with the plasmid pVU-DL106 at two hours allowed CPE to be detected at three days post-inoculation at passage three and the detection of mutated virus containing the x-gpt gene as determined by DNA-DNA 25 hybridization. A qualitative assay for B-galactosidase activity using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal) was used to detect mutated orf virus containing the B-galactosidase gene.

Example 10 - Construction of a vector plasmid suitable for the insertion of foreign genes into the region of the orf virus genome corresponding to the orthopoxvirus ATI-region 30

The intergenic region between the RNA polymerase subunit gene, rpo 132 and the open reading frame of the presumptive gene (H)IIL was identified as a suitable target site for the insertion of foreign DNA. The region is 90 nucleotides in length and lies between two converging transcriptional elements one of which, rpo 132, is an essential gene. A plasmid, PB-23\Delta Sal, which contains a sequence of 1.6 kilobases extending into the unsequenced region upstream of position 1 shown in the sequence illustrated in Figure 5 and terminating at the Pst site at position 178 was used as the template in a PCR cloning

A sequence of 1.0 kb was amplified from it using the primers zxs-1 GATCCCGCTCGAGAACTTCAA (forward) which is complementary to a sequence identified in PB-23ΔSal that contains an existing Xhol restriction site and zxs-2 GTCAGATCTATGCATAAAAATTTCGCATCAGTCGAGATA introduces a Bg/II, a Nsil and an Apol restriction site. The amplified fragment was purified by electrophoresis on a 1% agarose gel and digested with the restriction enzymes Xhol and Bg/II. The purified fragment was ligated then into the plasmid pSP-70 at the corresponding Xhol and Bg/II sites creating the plasmid pTvec1. This cloning step also introduced a poxvirus transcriptional termination signal (5TNT) into the vector.

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A second fragment comprising the sequence located between nucleotide positions 66 and the primers zxs-3 amplified with 1069 (Fig was GACATGCATCAGTGCCATGGAATTCTCGCGACTTTCTAGC (forward) which restriction zxs-4 **EcoRI** Ncol and introduces Nsil. GACGGATCCGTATAATGGAAAGATTC (reverse) which introduces a BamHI restriction site. The amplified fragment was digested with the restriction endonucleases BamHI and NsiI and purified in the same manner as the first fragment. The purified fragment was then cloned into pTvec1 which had been cut with Nsil and BglII. The resulting plasmid pTvec50 contains a series of restriction sites and a transcriptional termination signal which are available for further cloning steps. These restriction sites are Apol, Nsil, Ncol and EcoRI. The sequence of the primers, the restriction sites and the sequence of the modified intergenic region are shown in Figures 20A and 20B. The cloning steps involve in the construction of ptvec50 are illustrated in Figure 21.

A lacz gene under the control the orf virus late promoter PF1L was cleaved out of the plasmid pVUsp-PF2lac with EcoRI. The fragment was gel purified and ligated into the EcoRI site of pTvec50. Recombinant plasmids containing the lacz gene in both possible orientations were recovered and designated pTvec50lac-1 and pTvec50lac-2. The cloning steps involved in the construction of pTvec50lac-1 and pTvec50lac-2 are illustrated in Figure 22. This completed the construction of a transfer vector designed to introduce the foreign gene lacz into the intergenic site between the open reading frames of rpo 132 and (H)IIL shown in Fig 5.

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In this example the xgpt gene was not included in the transfer vector and consequently selection of recombinant orf virus expressing xgpt by growth in the presence of mycophenolic acid was not able to be used as a selection method. Virus recombinants were selected by using lacz expression as the primary method for identifying recombinants containing an insertion in the ATI region. The following variation of the method described in Example 8 was used.

Primary bovine testis (BT) cells were grown in monolayer cultures in Eagle's Minimal Essential Medium (MEM); (Sigma Cat. No. M0643) supplemented with lactalbumin hydrolysate (5 g/L) and 5% foetal calf serum. Prior to infection the cell growth medium was removed and the cells washed briefly with phosphate buffered saline (PBS) to remove residual serum. The cells were infected with orf virus, strain NZ-2, (moi 0.05 - 0.1) and the virus allowed to adsorb for 1 hour. Cell monolayers were washed 2 times with opti-MEM serum-free medium, (Life Technologies Inc, Gaithersburg, MD, U.S.A.) to remove non-adsorbed virus and residual foetal calf serum, and drained. A 1.0 ml volume of opti-MEM containing 10 μl Lipofectin reagent (Life Technologies Inc, Gaithersburg, MD, U.S.A.) and approximately 2.0 μg plasmid DNA diluted according to the suppliers instructions was added to each flask and incubated overnight. Following this overnight adsorption step, 5.0 ml of selective medium containing 2% foetal calf serum was added and the incubation continued until cytopathic effect (CPE) was observed approximately 3-5 days post-infection.

Cell monolayers were scraped from the flask, deposited in the bottom of a centrifuge tube by low speed centrifugation, washed with PBS and resuspended in PBS. The resuspended cells were subjected to three cycles of freezing and thawing and sonicated briefly. The virus titre of the harvested culture was determined and the material plated on fresh dishes of BT cells at a dilution calculated to give approximately 2000 virus plaques per dish. Sufficient material was plated to screen 50,000 plaques (25 dishes). The infected monolayers were grown under an a 1% agarose overlay and after 5 days incubation when plaques became visible, X-gal in a 1% agarose overlay was added to the dishes and incubated a further 12 hours for colour development to occur. At this stage, any coloured plaques which had appeared were picked and treated as described in Example 8. Further purification of the recombinant virus was achieved by repeated cycles of plating and picking single, coloured plaques until a pure culture of *lacz* positive virus was obtained.

### **APPLICATION OF THE INVENTION**

In accordance with the present invention there is provided a parapoxvirus vector, specifically an orf virus vector, containing exogenous DNA. The exogenous DNA may encode an antigen capable of inducing an immune response or may encode a heterologous polypeptide of which expression is desired.

The vectors of the present invention therefore have particular applications in the expression of heterologous polypeptides and antigens. The capacity to express antigens make these vectors particularly suitable for use in vaccines.

- Orf virus vectors have a number of advantages over vaccinia virus vectors. Orf virus has a relatively narrow host range compared to vaccinia. This reduces the vaccinia associated risks of cross-species infection and spread of disease. A further advantage is that orf virus is less virulent than vaccinia in man, reducing the risks of febrile response and lesions.
- 10 It will be appreciated that the above description is provided by way of example only and that the invention is limited only by the scope of the appended claims.

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## CLAIMS:

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1. A parapoxvirus vector comprising a parapox virus containing exogenous DNA.

- 33 -

- 5 2. A vector as claimed in claim 1 wherein the parapox virus is orf virus.
  - 3. A vector as claimed in claim 1 or claim 2 wherein the exogenous DNA encodes at least one gene product.
- 10 4. A vector as claimed in claim 3 wherein one gene product encoded is an antigen capable of inducing an immune response.
  - 5. A vector as claimed in claim 4 wherein the antigen is selected from the group consisting of HIV envelope protein, herpes simplex virus glycoprotein, *Taenia ovis*, *Echinococcus granulosis* antigens, *Trichostronglylus* antigens, *Haemonchus* antigens, *Ostertagia* antigens and combinations thereof.
  - 6. A vector as claimed in claim 5 wherein the antigen is a *Taenia ovis* antigen selected from the group comprising *Taenia ovis* 45W, 16kd, 18kd antigens and combinations thereof.
  - 7. A vector as claimed in any one of claims 3 to 6 wherein the exogenous DNA further encodes at least one product which is a biological effector molecule.
- 8. A vector as claimed in claim 7 wherein the biological effector molecule is selected from the group comprising γ interferon, IL-1, IL-2, IL-1β, IL-4, IL-5, IL-6, IL-12 and combinations thereof.
- 9. A vector as claimed in claim 8 wherein the biological effector molecule is selected 30 from the group comprising IL-1, IL-2, IL-12 and combinations thereof.
  - 10. A vector as claimed in any one of claims 3 to 9 wherein the exogenous DNA further encodes at least one peptide moiety expressed as a hybrid or chimeric protein with a native virus protein.
  - 11. A vector as claimed in any one of claims 1 to 10 wherein the exogenous DNA is incorporated in one or more non-essential regions of the virus genome.

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- 12. A vector as claimed in claim 11 wherein the non-essential regions are selected from the non-essential regions identified in accompanying Figures 2, 3, 5 and 7.
- 13. A vector as claimed in claim 11 or claim 12 wherein the non-essential region is from
   5 nucleic acids 11 to 16 in the sequence of Figure 5 or from nucleic acids 2226 to 2286 in the sequence of Figure 9.
  - 14. A vector as claimed in any one of claims 1 to 13 wherein the exogenous DNA is under the control of a poxvirus promoter.
  - 15. A vector as claimed in claim 14 wherein the poxvirus promoter is an orf virus promoter.
- 16. A vector as claimed in claim 15 wherein the orf virus promoter is selected from the group consisting of E1L, F1L and F3L as set forth in Figure 10.
  - 17. A vector as claimed in any one of claims 3 to 16 wherein the exogenous DNA further encodes a reporter gene.
- 20 18. A vector as claimed in any one of claims 3 to 17 wherein the exogenous DNA further encodes a selectable marker.
  - 19. A fragment or variant of a vector as claimed in any one of claims 4 to 18 having equivalent immunological activity thereto.
  - 20. A vaccine comprising a viral vector according to any one of claims 1 to 18 or a fragment or variant thereof as claimed in claim 19.
- 21. A vaccine as claimed in claim 20 which further comprises a pharmaceutically acceptable carrier and/or adjuvant therefor.
  - 22. A host cell incorporating a vector as claimed in any one of claims 1 to 18.
  - 23. A host cell according to claim 22 which is a eukaryotic cell.
  - 24. A host cell according to claim 22 or claim 23 which is a bovine testis cell or an ovine testis cell.

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25. A method for producing recombinant parapoxvirus vectors comprising transfecting a vector of any one of claims 1 to 18 into a selected host cell infected with orf virus; selecting a recombinant virus; and optionally purifying the selected virus.

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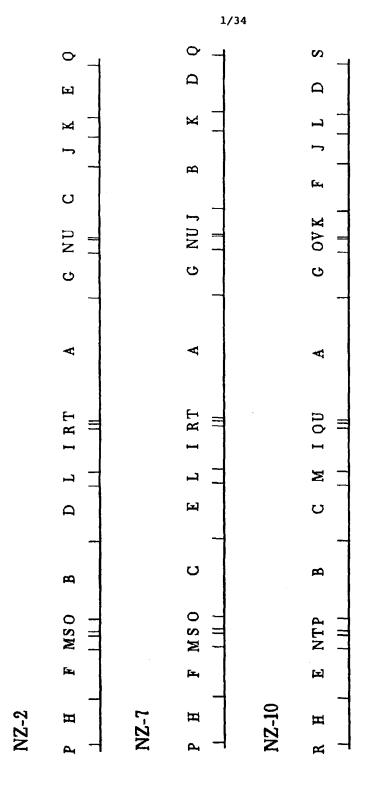


Figure 1. KpnI Maps of Orf Virus Genomes.

Figure 2. Nucleotide sequence of part of the KpnI E fragment of orf virus strain NZ-2.

	-	Ξ-	21	31	S	5] 6	61 7	71 8	18	16
	GGGCTTCCAC	GCGCTGCTCT	סמפרדונכאב מכמכדונרים בממחברבאב מחיכדנסומב דנמכמכמממ ככדמכמאכנם בידיננממכים במכחחוכד למכנמכאככ באדכמאמאב	genereoree	TCGCGCGGGA	CCTGCGACCG	ctrccsacts	cocororcer	CGCCGCACCC	CATCGAGCAC
101	1 111		121 13	131 141	11 151	191	171	181	161	
	، حىدەدەدەد	, הפדמבמברפר	enegeocogo hogracacos cancareme hecactada haanaceae eacadacace eccaneacos ecacaenere enanaceae aceasaaa	TCCCGCTGGA	TAVATGCCCC	CGCGGACGCC	CCCGACGCCG	CCGCACTCTC	CTGAGCCCAC	9299292229
201	1 211	1 221	231	241	1 251	1 261	1 271	1 281	1 291	***
	اددوووصدود	TOTACGACGT	כנסססרונסב וסואכסאנסו בחירכונסנס בשבורנדוסב מכנססרוסב במכנסכנסם הככנסכנה בסכנסכנים במכנסנדים במכנסנסב מוססחסכס	caerrecrae	GCCGGCTGGC	دودودودودو	GCGCCGGCCT	cogcagaana	caccaracac	gradaracad
301	116 1	1 321	וֹנוּב בּיוֹנוּ	1 341	351	1 361	175	381	391	<b>#4</b>
	receceece	CCTGCGGAAC	ידפרקכספכנס לכידפכסמאב ליפכמאפכיאם להסתפכימא לכסכיהמכמב מכסמאכסכסם לכסמכסכמר למכמכידאמכב לככמכמכסכ ליכמכפאמאכ	TGGTGCTGAA	CCGCTGCCAC	GCGGACGCGG	ccacacacat	CGCGCTAGCC	Teccocccc	TCGCCGAGAC
401	i (11	1 421	11 431	1 441	1 451	1 461	471	1 481	1 491	<b></b>
	GCTGGCGGAG	, כדכככםככם	פרדופסרסקאס לרבכבסבסבס לספאבאאסכד לכבבכהדכסכם לדכהאסרדסם מכסדסמאכרכ למאסראכככם מאסרדמאכסב לספאבבבככ	כפככפוכפכפ	CTCGAGCTGG	GCGTGGACCC	כפאפכאכככפ	GAGCTGACGC	CGGACCCCGC	CTGCGCAGGC
501	511	1 521	1 531	1 543	1 551	561	125 1	185	1 591	<b></b> -
_	GAGAGCGCAC	TCCCACAGAA	GAGAGCGCAC TCGCACAGAA CATCGACATC CAGACGCTGG ACCTGGGCGA CTGCGGAGAC CCCAAAGGCC GCCGACTGCG CGTGGCCTG	CAGACGCTGG	ACCTGGGCGA	CTGCGGAGAC	CCCAAAGGCC	GCCGACTGCG	cereceere	GTGAACAGCG
601	1 611	1 621	1 631	1 641	651	199	671	681	691	
_	GCCACGCGGC	CGCGAACTGC	פרכאכפכפס לימכמאורים במפרוכפכפר מכפורסכמאם לפנככונאכם במככמכמום לכמכשמככם לכאכפכרני	GCGTGGCGAC	cececteace	сессесетес	CCGCGAGCCG		מבפפאפפטרם מבאכפכנטכב	פריספכנים
701	111	127	1 731	1 741	151	761	771	781	191	
-	GTGGACGCTG	crecresess	פוספאכפכדה לומכרקפפכםם וספככםכםמד מאכםפופכדכ ממכמוסמוכם כממוזוכאכד	GACGGTGCTC	ממכמדממדממ	cogramcaca	פנדפנפנפנ	cccreceae	TACGCTACCG CITCGCGCGG	rrcecade
801	811	821	831	841	851	861	•	VEGF-2 →	881	891
-	ودووددودود	TGCGCGCGTA	CCGGCCGCGC TGCGCGCGTA GCCGCGCAAA ATGTAAATTA TAACGCCCAA CTTTTAAGGG TGAGGCGCCA TGAAGTTGCT	ATGTAAATTA	TAACGCCCAA	CTTTTAAGGG 1	rangececk		COTCGGCATA CTAGTAGCCG	TAGTAGCCG
106	116	921	186	941	156	961	11.6	186	166	        
_		CCAGTATCTG	CCAGTATCTG CTGAACGCGG ACAGCAACAC GAAAGGATGG TCCGAAGTGC TGAAAAGCAG CGAGTGCAAG CCTAGGCCGA TTGTTGT	ACAGCAACAC	GANAGGATGG	rccgAAGTGC 1	GAAAGGCAG	GAGTGCAAG	CTAGGCCGA	TOTTOTTO
1001	101	1021	1 1021 1031 1041 1051 1061 1071 1081 10	1041	1051	1061	1071	1081	1001	991
	TGTAAGCGAG	ACGCACCCAG	GCTGACTTC	דכאסכמסידים אתכינמכנכה מוסדיתמחות מתומכתתכ ממכממוככת מכתתכמתכת	AACCCGCCGT C	TGTCACGTT O	ATGCGATGC G	GCGGGTGCT G	CAACGACGA	GAGCTTGGAA
1101	1111	1121	113	1141	. 25	1162	1171	1181		
<b>-</b> `	TGCGTCCCCA	CGGAAGAAGT	TECETECECE COGRAGAGT AAACTEGTA ATGGAACTEC TGGGGGCGTE GGGCTECGGT AGTAACGGGA TGCAACGTET GAGCTTCGTA GAGCATAAGA	ATGGAACTCC	ragacacare	1960TCCGGT	GTAACGGGA T	GCAACGTCT G	AGCTTCGTA G	AGCATAAGA

Fig 2.

: :

ANGCOATTO TAGACACA TTCACAACCA CGCCACCGAC GACCACGAA GACGCCGGTA GACGCGGTA GACTTTTTA TGGACGCGCA ATCCAAACGA

1301 1311 1321 ITR junction 1351 1351 1371 1381 1391

TOATGCGATC AGGTCATGCG GAAGAAGGCG CCACGGAGCA AAGTGAAAA GGACCGCCTA GCAGTCGAGA CCCTCCGGCC GCAGCCGCGG ACACCCCACA

1401 1411 1421 1431 1441 1271 1281 1291 CCCGCCTTCC ACCCGCCAGA CGCCAACACC GCAGCCAACA AGCATCG Figure 3. Nucleotide sequence of part of the KpnI D fragment of orf virus strain N2-7.

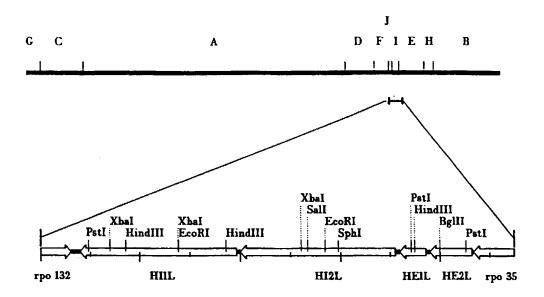
	-	ii	21 3	) ic	ii	ų ts	1.9	1.i e	9 19	76
	GGGCTTCCAC	deservences	מממנודנכתב  ממסנומרוכה  נספתמכוכתב  מתכרכפתמב  רכמכמכמספת  כנומכמתכה  נודניכמכנים  כמכמוסונני   למכנמנתכה  בתכמתכה	GGTCTCGTGC	TCGCGCGGGA	cracance	crrccscrs	cecererecr	CGCCGCACCC	CATCGAGGAG
101		iii ii	121 131	143	151	1 161		171 181	191	
	رىدەدەدەھە	restacece	eressesses restruceses cancerere recogerada hanarecese rechances eccanoses carteresta hacecadese desessess	TCCCGCTGGA	TAAATGCCGC	TGCAGACGCC	CCCGACGCCG	CACTCTCTG	AGCCCACGCC	0000000000
201	, 211		221, 231	11 241	1 251	1 261	1 271	71 281	1 291	<del></del> -
	GGCTCGCTGT	ACGACGICIT	oscroscrar Acancarerr cercococoe mecracoce ascrosces acocococos coscercos coscercos corecocaro asrocostos	TTCTGCGCC	GGCTGGCCGC	ರವಲವವವಾದ '	ceecereee	, ೧೮ <b>೦೦೧</b> ೧೮೦೦೦	corececto	GGTGCGGTGC
301	31.	1 321	12 33	341	1 351	1 361	1 371	381	1 391	<del></del> -
	GCGGTCGCCT	GCGGAACTGC	פכמפרכסככד מכמס <b>אור</b> דוסם מאכרזסמדסם דמכדמאולכם כדמככאכמכה מאכמכנממכר ממכמכמכדנם כמכדממכנדכ כמכממכמדם מכממאאנככ	TGCTGAACCG	CTGCCACGCG	GACGCCGGCC	6656565756	cacreacere	cecececae	GCGGAAACGC
10\$	£13	1 421	23 431	1 441	1 451	1 461	1 471	1 481	1 491	
	TGGCGGAGCT	ರಿದ್ದಾರವಾದಿದ್ದಾರ ಕರ್ನಡಿಗಾಗಿ	TOCCOGNICT GCCOCOCOCO DACANGETCG CCOTCOCOCO CONGCTNOGC GTGONCCCCO NGCNCCCGGN GCTGNCGCCG GNCCCCGCCT GCGCGGCGN	ರದರ್ಶದರ್ವದ	CGAGCTAGGC	GTGGACCCCG	AGCACCCGGA	GCTGACGCCG	GACCCCGCCT	GCGCGGCGA
105	1 511	1 521	11 531	3 541	1000	198	1 571	1 381	1 591	<b></b> 1-
	GAGCGCGCTC	GCACAGAACA	GENENGANCA TEGACATECA GNEGETGGNE ETNGGEGAET GEGGEGACEE CANAGGEGEGE ÉGNETGEGGE TGGEGETGGT GANCAGEGGE	GACGCTGGAC	CTAGGCGACT	GCGCCGACCC	CAMAGGCCGC	CGACTGCGCG	TGGCGCTGGT	GAACAGGGG
109	1 611	1 621	1 631	1 641	169	199	1 671	189	1 691	
_	CACGCGGCCG	CGAACTGCGC	כארמכהמכנס לכמאתידמכים לפירכמכסכים לידססכמאכרם למכיהמאכשה לכמכסיומכנה לכמאמבכשה אכמכניוכמה למאמשהמשם אכמכנמכהי	GTGGCGACCG	cecreacece	ccccareccc	CCGAGCCGGC	Acaccreac	COAGGGCGGC	Acececer
107	721	721	1 731	741	182	161	177	1 781	191	
-	GGACGCTGCT	GCTGGCGGTG	ecraecadre accacaeras consercas consareaca Armicacrae hacoccacos actaasaara comminan Acicaaate	cogrected	COTGOTGCCA	Arraceree	recoccacec	GCTAAGAATA (	CGCTTTAGAT	ACTCAAAGTC
108	8118	82]	1 831	148	158	861	871	887	1 891	
<b>-</b> -	TATCCAGACA	CTTAGAGTOT	tatccagaca cttagag <b>tot aactttgagt aaaaaa</b> tgta aatactaacg ccaaaatttc gatagttgtt aagcaatata taacattttt aaaagtcat	AMAMATGTA	AATACTAACG (	CANANTTEC (	SATAGTTGTT	AAGCAATATA 1	TAACATTTT ;	WACGTCAT
106	911	126	1 931	941	951	196	971	1 981	166	
1001	VEG CACCAGCATG	VEGF.7-	VBGF-7-*	AGTTGTTGTT (	GCATTGTTAA 1	FATGTATGTA 1	FAATTFGCCA (	GAATGCGTGT C	TCAGAGTAA 1	TGATTCACCT
1011	CCTTCAACCA .	ATGACTGGAT	CCTTCAACCA ATOACTGGAT GCGTACACTA GAGAAAAGTG GTTGTAAACC TAGAGATACT GTTGTTTATT TGGGAGAAGA ÅFATCCAGAA ÅGCACTAACC	GACAAAAGTG G	gttgtaaacc i	   rgagatact     reference   reference	TTGTTTATT T	roggagaaga A	TATCCAGAA A	GCACTAACC
~ •	TACAATATAA	TOCCCGGTGC	TACATATAN TOCCGGGTGC GTAACTGTTA AACGATGCAG TGGTTGCTGT AACGGTGACG GTCAAATATG TACAGGGGTT GAAACAAGAA ÁTACAACTGT	AACGATGCAG 1	regreerer A	ACGGTGACG G	TCAAATATG 1	CACAGCGGTT G	MAACAAGAA A	TACAACTGT

Fig 3.

1201	1 1211	11 122	123	1241	1251	1 1261	1 127	1 1281	11 1291	
-	AACAGTTTCA	Arcagettca gtarccogog totctagetic gectogeract areageges tatceacea coefecaaga ataageste cagarcacac aaasegegae	TOTOTAGETIC	GTCTGGTACT	AATAGTGGTG	TATCTACTAA	CCTTCAAAGA	ATAAGTGTTA	CAGAACACAC	AAAGTGCGAT
1301	1311	1321	1 1331	1 1341	1351	1361	1 1371	1381	1 139	<del></del> 4
-	TCTATTGGTA	TOTATTGGTA GANCAACGAC AACACCTACG ACCATAGGG AACCTAGACG ATAACTAATA ACAAAAATG TITATTITG TAAATACTIA ATIATTACAC	AACACCTACG	ACCACTAGGG	AACCTAGACG	ATAACTAATA	ACAAAAATG	TTATTTG	TAAATACTTA	ATTATTACAC
1401		1411 1421 1431 1441 1451 1461	1431	141	1451	1461	1 1471	1 1483	1491	
	ACTITACAAT	ACTITACNAT NATCICANNA ATMATTGCG TGCCGGGACG GCTGCAGCTG GTGACGCTGC TGTGTCACAC ACTGCGTAIT CGATTCAAGT TCACTAACGC	ATAMATTGCG	TGCCCGGACG	GCTGCAGCTG	GTGACGCTGC	TGTGTCACAC	ACTGCGTATT	CGATTCAAGT	TCACTAACGC
1501	151	1 1521	1 1531	1 1541	1551	1961	1 1571	1 1581	1 1591	
	CACTAAACTA	CACTARACTA GITGEGGEG ICCGAGTOTF ARCCGIACGE CAAACTAACA ICTTACCTGE CCGIGACAAG AACTAAAACT IGAACCACAI ATTITIAAAG	TCCGAGTGTT	AACCGTACGT	CAAACTAACA	TCTTACCTGT	CCGTGACAAG	AACTAAAACT	TGAACCACAT	ATTTTAAAG
1091	161	1 1621	1631	1 1641	1651	1991	1 1671	1 1681	1 1691	
— <sup>-</sup>	TATATTTAAC	TATAITIBAC AAAACACTC ACACTCACAC AATCAIAAAC ACCACAACCA CAACCAAACA CGCAIGAGAA TTAAIATTCI IACITAICCG IAACACTCIA	ACACTCACAC	AATCATAAAC	ACCACAACCA	CAACCAAACA	CGCATGAGAA	TTAATATTCT	TACTTATCCG	TAACACTCTA
1701	171	1 1721	1731	1741	1751	1761	1771	1 1781	1 1791	
<b>-</b> `	TGCTGTACAT	TOCTOTACAT CAACOCATCA GAGCAGTCTG AGTCTGACTA ATGGCGGCAA ACGGGAACGC AGGCGCGACA TAATCACTGA GAATCTCCGC AGCAACCGCT	GAGCAGTCTG	AGTOTOROGEN	Argeogecal 1	ACGGGMCGC	AGGCGCGACA	TAATCACTGA	GAATCTCCGC	AGCAACCGCT
1801	181	1 1821	1831	1841	1851	1861	1871	1 1881	1891	
_~	CAAGGACATC	CAAGGACATE TETAGEGETA ACGGETGTIT GICATICCEE EGIGIGITEA TETEACAGA CATIGIGACE GICGCANAGE ACACATICAA AGIGEGGAL	ACGCTGTTT	Greatress (	cerererrca 1	TCTCACACGA	CATTGIGACC	GTCGCAAAGC	ACACATTCAA	AGTGCCGCAT
1901	1161	1 1921	1931	1941	195	1961	1961	1981	1991	
	GTGGAAGAAT	GIGGAAGAI ICACCGICGA GACACACAC ATANTIANAC ANGAICAGIG CATANGAGAG ATTAGCAITC IACAGCACAC CACGIGGGAA IACGGACCIC	GACACACACC	ATANTTANAC )	AGATCAGTG C	CATANGAGAG	ATTAGCATTC	TACAGCACAC	CACGTGCGAA	racedacere
2001	2011	1 2021	2031	2041	2051	2061	2071	1 2081	2091	
_ ~	GTAATTGTTT	GIAATIGITI AGACIAGAAC ACCICTGGIC IAAACAACAT GICCGAICIT AGAACAGAGI TIAIGACGCA TAIGIAACIG IGIICITIAI GIAGAAGITA	ACCTCTGGTC	TAMACAACAT C	STCCGATCTT )	NGAACAGAGT	TTATGACGCA	TATGTAACTG	IGTICITIAT (	STAGAAGTTA
2101	2111	1 2121	2131	2141	2151	2161	1,712	2181	2191	
	rcttttage	rettraner exercectra rettagarga grianacard acardatera igrefecee degecoede dogecoerco degecoege iderocede	TCTTAGATGA	GTTATACATO	CATGATGTA 1	יסופינים	202002000	GGGGGGTCG	2000000000	recrecede
2201	2211	1 2221	2231	2241	2251	2261	2271	2281	2291	
_	5000000000	ספרספסברבים במפונספר שמרושנונים בנפרד-פרשם כרפנספכנים מכסכרבים בכספרסבים אפרספרכנים בניביבנים פרבנים בניביבנים	6901060606	             		CGGCGGGGT	AGCGGCCCGC	בכפכככפפפכ	CCCCCCCCC	SCCTTGCCC
2301	231	2321	2331	2341	2351	2361	2371	2381	2391	
O	JGGACCAGGC	COGRECAGO OCCACOGAGO ANGTONNA AGENEGEET AGENGTEGAG ACCTECEGE CGCAGEGGE ACACECEARA ECCGCETTEC ACEGECAGA	AAAGTGAAAA	AGGACCGCCT A	GCAGTCGAG A	וככבבככפכ	20CAGCCGCG	ACACCCCACA C	ccecerrec A	CCCGCCAGA
2401	2413	2421	2431	2441	2451	2461	2471	2481		
O	CCCAACACC	פפנמאמתכי אמאנכנאתנא אמנאומנאני ברובשננפנים באמנותביב מבימנמרים במוממומנים בוממנותב אמנים ביוממנותב ביוכחנים במ	AGCATGCACC (	ومتحودودو ا	AGGCTGCTC G	מכפכפכזבפ	sarcarece i	rasserree i	cereacea	

Fig 3.7

FIGURE 4. HindIII Map of Orf virus NZ-2 showing the location and orientation of the reading frames for the putative genes, rpo132, (H)I1L, (H)I2L, (H)E1L, (H)E2L, and (H)E3L (rpo35).



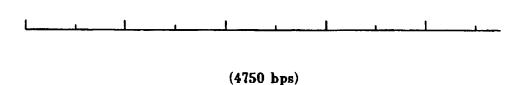


Figure 5. Nucleotide sequence of genes HIIL, HI2L, HEIL and HE2L from orf virus strain NZ-2 showing potential insertion sites.

	-	~~	21 3	17	15	6	_		181	
٧	ő	AAACGCGCGG	<b>9</b> 292252592	GAC <u>IGA</u> IGCO AAACGCGCGG CGGCGCGGG ÁCTIAGCTIA ÍCICGACIGA ÍGCGAACGCG CGACCICICG CGACITICIA GCITCYCAGA CIGAIG <u>CIA</u> C	rcrccactga	recgaacece	CGACCTCTCG	CGACTTTCTA	GCTTCTCAGA	CTGATGCTAC
	1=	121	131	11 141	151	161	171	181	1 19	
	2030	cerecreece	CATATCGCGG CGTGCTGGCC CCACCACCAG GGCTTCTCGC	ATATOGOGO CONOCAGOGO CONCONOCAGO GOCTTOTOGO CONOGOCTOS COGOGOCTOS COGOGONOGOS COGOGOGOGO COCONOTOS CONOGOGO COCONOTOGO CONOCAGOGO COCONOTOS CONOCAGOS COCONOTOS COCONOTOS CONOCAGOS COCONOTOS COCONOTOS COCONOTOS CONOCAGOS COCONOTOS COCONO	COTGCCTGAC C	GCGGGGCTGG C	CreceAcece c	CGCTGCAGTA C	Caragardac deoaggarga crocsaceca eceracaata dergeoega ececagrese	cccAGreec
	3116	CGCCGGGGGC	AGGCTCCCGT	cacachadra cacadagae Aggerecar canacacara ecocarana respondeda decagadaca cararachas figanahada 1111111111111111111111111111111111	CCGCGTCACC 7	TCGCCCGG G	GCCGCCGCA C	CGTGTGCACG 7	TCCGTCTTGT 11111111111111111111111111111	IGGAGACGAG
	67.AC	TGCCGCATGG	rererandra	CACCGGGRAC TGCCGCARGG TCTCTARGTG ATGCTCGAAG TGCTTGCCGGTT GGACTCGGAG CACGTTTTTG CTTCGGCTAA GGTTTTTTCT 411 411 421 421 421 431 431 441 441 451 451 461 461 471 471 481 481 481 481	rgcrrgcccg	CCATCCGGTT C	GGACTCGCAG C	CACGITITIC C	CTTCGGCTAA G	<b>36111111CT</b>
	GATA	GTAGCTTATC	CACGCGCTCG	AGAGGGGATA GTAGCTTATC CACGCGCTCG GGCAGGAGGC ACGGGAGCC ACGAACCCT ACTTTGAACG GGGTCACCTT CATGTTCCCG TCGTAGCGGT  111111111111111111111111111111111	ACGCGGAGCC 0	GTCGAACCCT A	ACTITICANCO O	GGTCACCTT	GATGTTCCCG	FCGTAGCGGT
<b>~</b> ~	GCAT   111	CCTGAGGTAG	GTTGTACCGT	CCCACAGCAT CCTGAGGTAG GITGIAGCOT EGGGGTCTGG GICTGICCAC ACTCTAAGCT ITTGGCTACA GCGGCCGTCG IACGTAAGAC GGTCTCTACG	   STCTGTCCAC   	ACTCTAAGCT 7	rttcgctaca q	10000001CG 1	TACGTAAGAC C	GTCTCTACG
<b>y</b> -	717,	TYCTGCTTA 1	Torrorroce	CICGIAGIAG TITCIACITA TOTIGIAGG GICICCAIGC ICGIAGIAGI AIAANCGIA CGCGCOTGGC IIITITAAAG CGITTICGIC GIIGCIGAG 21. 71. 72. 72. 73. 74. 74. 75. 75. 76. 76. 77. 77. 78. 78.	CCGTAGTAGT A	NTABATCGTA C	1600000000 T	TTTTTAAGT C	CGTTTTCGTC G	TTGCTGACG
55 -	- 1004 - 111 - 111	CGGGATAATA	GGATATCCTA G	TCTATCACOT COGGATAATA GGATATCCTA ACTGCACTAC AATCTATAGT ATTTGGTCTA GTAAGCTGTT CGAGATCACC TTGTTCATCA TGATCTACTG	LATCTATAGT A	TITIGICIA 9	TAAGCTGTT C	GAGATCACC	ITGITCATCA I	GATCTACTG
<b>~</b> ::	CAC (	######################################	TGTTCCGACG	ATTGTACAC GGCACCGTCG TGTTCCGACG GACGTATGAC TATGTCCATG GTAAACGATG TACCCACTTT GGAAAACGTA TCCCATGCAG TAAAGCATAG  11. 11. 11. 11. 11. 11. 11. 11. 11. 11	ATGTCCATG G	TAAACGATG 1	ACCCACTT 6	GAAAACGTA 1	rccargcag T	AAAGCATAG
U	101	TABACTCAG (	GAACACTCAT	TECGTECATT ATAMACTEM GANCACTEM ANGMANTEGA ANTETOTICAN GITTITEGAA CACCACTITI ACMIGGICIT IGICACGANG ACATISCEG 11:1111111111111111111111111111111111	ATCIGIGAA G	TTTTCGAA C	ACCACTITT A	CATGGTCTT 1	GTCACGAAC A	rcarrecce
	CAG	CATGAATTG	AAGGAACGCT	THIRCTICAS ACADGANTIS AAGGACGCT AAGAACTIC TISTICTIC AGAACCTIT CCATTATACS ICCATCCAST TICTASAAT CTATATATC	rgrrrcrrc A	TGAATCTTT C	CATTATACG T	CCATCCAGT 1	TCTAGAATT C	TATATATGC

Fig 5. 1

	<u>د</u> :	<b>€</b> -	£ :	ပ္ပ -	8 -	리는 !	<b>Ļ</b>	<b>4</b>	υ <b>-</b>	<b>«</b>	o -	<b>6</b>	•
<u>-</u> -	TTANAAG 	GGTTACAT	Arrecerr	CGACATTA(	TAGAGACTO	GTTAATCCTT	AAGACGTGT	ATCTTTGA 1111111	1111111 1111111	GTCTCCCA	TAGTCCAA	Taggaacti 	AGTTATCCC
1191	CCATTGCGT	CATTGGTAAC	CCTAACGTA	197CTACCGG	CACGGAACT 1	ACTTCGTAT (	Grerchece A	ATTACCTGT	TATCGITA C	ATTGTCTA T	TAATGTTG A	CCATGGAT C	hetagama trgaggacta garataggee trecamatee tggatgataa accamaggea atgenetaga gtggacateg teactgtete tagitateee
118	3GCAAGAGTG	AAATTGGTT (	TACATGTTC 1	AGATTTACC G	GAATACACG     	AATTATCCC A	2ATAACGGG C	rrgrrrcrc C.	CACTGAGG TO	mccrrrrc r	TATTGTAC TC	TACGIAIC TI	CGACATCG TC
1711	TTITIGGARC GACCCOGRAC GACCAGRACA TOGGAACTCC GAAARARA GCGGGGTTTO AGRACCAATG GGCAAGAGTG CCCATTGCGF TTAAAAAGTC  2221, 1221, 1221, 1231, 1241, 1241, 1251, 1261, 1271, 1281, 1281, 1291	TTGACAAAAA AATGCAGTIT TTGTGTGGAT AACTGAGTT GGACTACGTT CGTGGACATC GTACATGTC ATAATTGGTT CATTGGTAAC GGTTACATGA 	CCCGICATTA TCTTTTAM AATCATAAA TACAGTITGC CTAAAGTCGA AATATGTATA ACGTTAATT TIACATGTTC TCCTAACGTA ATTGCGTTTT CCCGICATTT TACATGTTT TACATGTT TACATGT TACATGTT TACATGT	TACTTAGCCA TECTCGTCT ACADANTCT TACGATACAT AGGATTTCTC TCTAGGTATC TTCTAAAGTA TACATTTACC GGTCTACGG CGACATTAGC	ecatotata geaggagca, gotgtatota togtgbata angtotoga taagottiot giteticiote ggaltacacg ácacggaact tagagactgg 	+-IIL ***********************************	ELACCAGAT ÅGCACCGCTC CTTCCTCTCC ÅCCACGTACT ÅTCTANAGGA ŤACOTGTAAG ĠGTAATGTCT ĠGATAACGG ĊGTGTGAGCC ÅAGACGTGTT 	CCCACTACT G	CASTATCCA TO	TCTTCTGTA CC	CCTACTTCT TC	3trGCCTAC rg	PGCACTACA GT
911	acresorms	COTGGACATC C	AATATGTATA J	rcracgrare	FAGCTTTCT	+-IIL ***********************************	ACCTGTAAG G	CGTTGTCGA T	TTAAGTCTG T	CTCTAAGCA T	CTCGTTATT TO 1111111111111111111111111111111	TTGACATCG TC	CCAAACGCA AT
1151	GAAATATATA 11111111111111111111111111111	GGACTACGTT C	CTAAAGTCGA	AGGATTTCTC 1	Argrereara 1	Accorder J	TCTAAAGGA †	GAGTGCTAG A	AATTAGGTC	ATAAACCTG T	rregerere 6	   retricate A   received   2351	SCATGATAL A
1141	TGGGAACTCC	AACTIGACIT O	TACAGTTTGC 111111111111111111111111111111111	FACGATACAT	rcarcarara J	TAGGAACCA 1	CCACGTACT A	CTAACTACC   	CEAACGGCE 1	CANATANCC C	AGTOTGTOC C	ATATAATCG G	CCANATCC TO
1133	CACCAGEACA	Treforcear 1111111111	AATCATAAGA	ACAAAATCT	GCTGTATGTA :	AGACTTGAAC	Treerere A	darccacrrc 1	GCCAAATCT T	Grthagign C 111111111111111111111111111111111111	Grrotacor A	Tricrigia &	MANTACGCC TO
1123	GACCCCGTAC	AATGCAGTTT 1321	TCTTTTTAAC	rregregrer 11111111111111111111111111111	GCAGGAGCAA	PTCAACCAA 1	3CACCGCTC	CCCACCAAC	GITAGICIT T	GGAACTECC T	TTACGCATC C	STTTICATG A	PCAGGACTA G
1111		TTGACAAAAA	CCCGTCATTA	TACTTAGGCA TICGICGICT ACAAAAATCT TAGGATACAT AGGATTICTC TCTAGGTATC TICTAAAGTA TAGATITAGC GGICTAGGG CAACAITAGC		TGCCAGTGTC TTTCAACCAA AGACTTGAAC CTAGCAACCA ACGCGTTGTC ACTCTCCATT TATAATTAAA TAATTATCCC AACTTGGTAT GTTAATCCTT    III   172    173    174    175    176    177    178    179	ATTACCAGAT AGCACGGTC CTTCCTCTC ACCACGTACT ACTALAGGA TACCTGTAAA GGTAATGTCT GGATAACGGG CGTGTGAGCC AAGACGTGTT 	ATGOTOTET CECEACEAE GOTECACTTE TETAACTACE GOAGTGOTAG ACGITGTCGA TECCACTACT GITGITTGTE CATTACETG AIETITGAA  1911 1921 1921 1931 1941 1941 1951 1961 1961 1971 1981 1981 1991	GCGCAACAA TOTTAGTOTT TGCCAAATOT TOTAACGGCT TAATTAGGTC GTTAAGTCTG TCAATATCCA TGCACGGAGG TGTATCGTTA CAGGTTCCC 101 11 11 11 11 11 11 11 11 11 11 11 11	CAACTTIGG AGGAACTTC TOTTIAGIG CANAIAAC CARAACCIG TCTCTAAGCA TICTTCIGIA CONGCITITG TCATGICTA TGTCTCCCAA	AAACCTGTGA TTFTAGGCATC CGTTGTAGGT AAGACTGTGC CTTGGTCTC GCTCGTTATT TCCTAGTTGT TCTATTGTAC TGTAATGTTG ATAGTCCAAG	TAATAGCCAC TGTTTTCATG ATTTCTTGTA AATATAATCG GTGTTTTAATT ATTGACATCG TGTTGCCTAC TGTACGTATC TTCCATGGAT CTACGAACTT	GTOTINGIALIA TICANGANCTA GANATACGCC TICCANATOC TGGATGATAA ACCAANGGCA ATGCACTACA GICCACATGG TCACTGTOTC TAGITATOCC
1101	1201	1301	10 <b>9</b> 1	1502	1601	1,001	1801	_* 1061	, too2	, 'C' 2101,	ر م - 1022	77 2301	_6

Fig 5.

-	AGTICCATAT	GAAATGTTTA	CTGGAAAATC	ATAATTTGCT	ATGATAGTGT 	rttgagcact	SCTTGGGTTA	TCANAGGCT	certate Certate	TTACCTGTT		ACACTCCGT		PEZL STTCCT <u>CTA</u>
2491	CATTGAACGC J	CTTATCAAAC G	ATGCTAACGT (	CATITITATA A	STAGCTATCC 2	TTCTTATGT 1	15GAAGGCC 0	GACCTATTA T	CAGCGGTTA TI	GTCTAACCG G	3491	CAGCCGCG TA	3591	CGAGTAAG TK
2481	AALATATCCA (11111111111111111111111111111111111	TAGTATCGAA C	AGCATCGGGC J	ACAAGAGACT T	rcrecreage c	STTGTCAAAA 3081	MTAGATITA C	AGGTATACA T	FIZE ATTATAATT AAATAATTAC AGAGGGGAAC ACAGGGGTA T <u>CTA</u> 11111 3151 3351 3381 3381	TTTCTAGTC T	3481	AGTTGGAGC T	3581	-ELL TTTCATCGA TATTGAGTCC AACGAACACA AACGAGTAAG TGTTGCT
2473	GAGTGTTAAA A	ACTACAAAAT T	TAGCTCCTCT A	CAATCCIATC	GGTTFACTAG T	CCGTGTTAT C	ACTCTTTAAA A	TAGTTCTTC T	AATAATTAC A	TCCAAGTGT C	3471	CACTTICTT T	. 3571	ATTGAGTCC A
2461	TOTATATGTT	CGGAACCGAT J	ACAAATCTA 7	AGTACATIGG O	GTTTAAAACC G	AATCTTCCTA (	GTCCACATA A	ACAACTTTT (	TTTATATT A	ACGTAGACT O	3461	ccrerre A	3561 ·	TTCATCCA T
2451	Transtore (	TAATGAATTC	CATGRAFIGE A	TCTCTCCACC A	CCATCTGTAT 0	ZATTAACAAA )	ACTGGATTTC 1		+121 AGCTTCTCC A	GAAGAGCTT T	3451	GCTTACGAG G	3551	TCTCCGTCA T
2441	**************************************	AAACCATCCA 11111111111111111111111111111	ACACATCTGT	ATTITIGGTCA	CAAAAATAAT	TGTAAACAAC 1111111111111111111111111111111	ACTITITIONA 3		GTCACTGAC T	TTGAGATCA A	341	TGTCTAACA	3541	CAATAGGTT T
2431	GATGCTCCAC	CGTATAACAC	TTTTTGTCAT	CGAACTGTTG	GALATTATAG	GACATTATTC '	TTTCAATAT 1	rrgcrffarg '	ACGTTGTA 3	TATTTTEE G	3431	Greenska C	3531	CAGGCTGAG T
2421	ARCAJCAGT CCITCITION GARGICCIA TOTITIANG TRACTOCIC TOTATAGET GAGIGITAA AAALATOCA CATIGAAGG AGITCAAAT 111111111111111111111111111111111	THOTHAFT TARGCAGAA GGTAFAAGAG AMGGAFCCA TAAGAATTC GGGAACGGAF ACTACAAAA TAGTAFCGAA CTFATCAAA GAAATGFFFA 2611 2621 2631 2641 2651 2651 2661 2671 2671 2681 2691	Credecorra focrecorar trensorar Acadaronar Casorarota Acadaarota facercorot Acareogogo Asgerande Credaaaarota (1908)   1908   19	ATTITITAS TOTOCIAGIA CGAACIQITG ATTITOGICA TOTOCICCACC AGTACATIGG CAAICCIATC ACAAGAGAC TATTITIATA ATAATITICT 2811 2811 2821 2831 2841 2851 2851 2861 2871 2871 2881 2891	ACAGAAGGTA TATGCCACAP GANATTATAG CANAANAA CCATCTGTAF GTTTAAAAC GGTTTACTAG TCTGCTGAGA GTAGCTATGC ATGATAGTGT 1111111111111111111111111111	Trocorogoc Artactroca Gacattare Totaaacaac Cartaacaa Arterecta Cogregata Gitgecaaa Giterrator Titgageact	TECRAGIANC CATATOTTAN TITICAANN ACTIVITOR ACTOCACAN ACTOCITAAA AANGATTAN CIGGAAGGC GOTIGGGTTAN SILL SILL SILL SILL SILL SILL SILL SIL	CTCCCTGTA TAGGCGCCC TTGCTTTATG TATTCCCGTA ACAAATCTCT TACAACTTTT CTAGTTCTTC TAGGTATACA TGACCTATTA TTCAAAGGCT 11.11.11.11.11.11.11.11.11.11.11.11.11.	TOTCCAOTT AGGSTTATA AAGSTGTAA AGTCACTGAC TAGGTTCTCC AFITA	TCTGTACAT C	3421	ATCAGTTAT '	3521	  ccrgtatca
2411					ACAGAAGCTA	TTCCTCGCC AREACTICCT GACATAITC TGIAAACAAC CAFTAACAA AATCTTCCTA CCGTTGTAT GTTGTCAAA GTTCTTATGT TTTGAGCACT  **********************************	TECGAGEAAC CAPATGETAT TETCAAATAT ACTETETATA ACTECACATA ACTECETAAA AAFAGATTA CIGGAAGGC GETIGGGTA	TCTCCCTGTA TAGGGGGGG TTGCTTTATG TATTCGCGTA ACAAATCTCT TACAACTTTT CTAGTTCTTC TAGGTATACA TGACCTATTA TTCAAAGGCT 3211 3221 3231 3241 3241 3251 3261 3261 3261 3261	FIGURE ANCOTITATA AACOTIGIAA AGTEACIGAE TAGETECICE ATTATAATE AAATAAITAE AGAEGGGAAE ACAGEGGTA TELAATATETET TOTAATATETET TOTAATATETET TOTAATATETET TOTAATATETET TOTAATATETET TOTAATATATATATATATATATATATATATATATATAT	GONGHANCCE GEOTOFACAT CTATETITICE GEOGRAPICA AGAAGAGCET FACGFAGACE CECCAAGEG CITICEAGE ISPOTAACCE GETACCEGET	3411	<del>acietecage aateagitat agititgiaa</del> et <del>gictaaca</del> agetiaegag gegetetiee aeaetiteit tagitegage tecageegeg tacaeteegi	3511	TOGITGANIT GCCTGIAICA TCAGGCTGAG TCAALAGGIT TTCTCCGTCA TTTTCATCGA TATTGAGTCC AAGGAACAA AAGGAGTAG TGTTCGTCIA
2401	2501	2601	2701	2801	2901	3001	3101	3201		-	3401	. • •	3501	

Fig 5.

Fig 5.4

orceacad georecado fracadada conascista acsecates sesassista eccadence dasseciste ecatesiste consistantes ceecorres cacarrana resessarer rerestavar eregiasacs secessaser senaceecae saacarses secesses seassaceae AUGOAGOGOC ACTICITIGOS CACGICGAAS GOOTOGIOST TGGGGTGGAA CAGGGGGGG TCCAGGGGG GGCGGGGGG CGIGGGGGG AACTCCAGGG 3741 3751 3761 3771 3781 3861 3871 3881 3851 GCAGAGGGG GACAGGGGT CCATCTTTA TGTGCAGAL TATTGTTG C 3831 4001 4011 4021 4031 3731 3801 3811 3821 372i 371i 372i

000

<u>8</u>\_



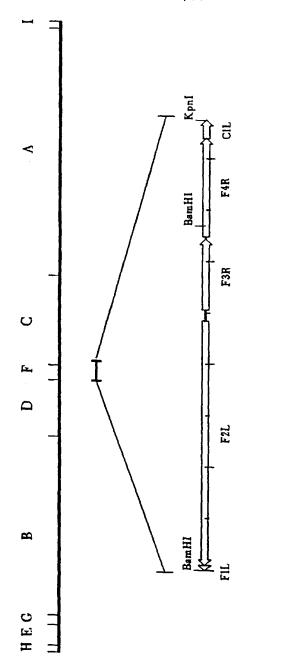


FIGURE 6. BamHI Map of Orf Virus NZ-2 showing the location and orientation of the reading frames for the putative genes FIL, F2L, F3R, F4R (topoisomerase) and CIL.

Figure 7. Nucleotide sequence of BamHI F and part of BamHI C from orf virus strain NZ-2 showing potential insertion sites.

ACAGGAAGAA GACGAACTCC AGTCCACGC GGTTCAGAGA GTCGCTGAAG TACACGAAGA CGTCGCTGTC CGGGAAGAAG CTCGCCGGA ACATGTTGTA 401 411 421 431 441 451 451 471 481 491

TOTAGGGGTT GOTGTGGTGG ACGCGGATGA GECGGACATG ATGTCCTGGA ACTCCGCGCG CGCGTCGGGGG CTCTCGGCGG GCGTCCGC 01 711 721 731 741 751 761 771 781 791 191 AGTGTACAC AGTGTACAC CTCGGTGAC AGTGTACAC CTCGGTGAC CTCCTTGCG CAGACGCGCG TCTTCACGAG cecandado anoncagos neaccedon anonome incacedece aconcacer encardas benecande acareacan binameano

Fig 7.

ANGOGIIGGC TOTGCGAGAA GTAGCTGTAG GGCTCGCTGA GGAAGATGGA CTTGTTGGTC GCGGGCACCA CCACGCCGGC GCGGGGCG GACGCGTCGG 1801 1811 1821 1831 1841 1851 1861 1871 1881 1891

AGOTTGAGCT TGCCCCGCGA GACCGGGATG CCGALGTA CGCGAACTCG AGGTACTTCT TCGAGAAGCG GATGCGGTCC AGGTTCTTGG 1901 1911 1921 1931 1941 1951 1961 1971 1981 1991 1991 AGAGGATCT AGAGGATCT GCGCACGGCG GGCTCCCCCGAA GGCGGTGCGC AGATCGCTGG TGCGCTGTAC 2201 2211 2221 2231 2241 2251 2261 2271 2281 2291 CACGANGNTO GACTTOTTOT COGCONTON OTCOGNOTING GACTTOGIGG COGNOTICGG CICCOCCAIG TACGCGCGGA TCTTCGGCAC GAIGCTCGCG 2301 2311 2321 2331 2341 2351 2361 2371 2381 2391

Fig 7.2

2401	1 2411		2421 2431	1 2441	11 24	2451 2461	51 24	2471 2481	81 2491	1
	 Aggatggact	CCCTGGAATC	- F2L ***********************************	CGGCAAGGGC	GCGCGAGACC	GTCTCAAAAC	TGAMTCGTA	TAMACTETTA	AAAAATCGGT	ATTGAMGTA
2501	1 2511	1 2521	21 2531	1 2541	11 2551	51 2561	11 2571	71 2581	31 2591	
-	CGCACCACCA AATAAAGCGT CC	AATAAAGCGT	ccaccacca aataaacce ccaccacce catectice teccartea aaateacca ctetteage tecaccace tecacacte cgacgatete	F3R→ CATGTCTTCG	 TGGCGACTCA	ANTGAGCAN	 GTGTTCAGGT	 TCCAGCAGCG	 TCCAGACTCT	 cgaggatctg
2601	1 2611	2621	2631	1 2641	1 3651	1 2661	1 2671	1 2681	11 2691	<b>z</b> -
-	CGTAATCGTC	TTCGCTCCGA	cgraancone incoencesa secentesee laceannece lasasceces esaceacere inceceases sesassasts ichsaacare sacesseeen	AACGATTGCC	AAGAGCCCCG	CGACGACCTC	Trecesage	GCGAGGAGTG	TCTGGACATC	GACGGGCCCT
2701	1 2711	2721	12 2731	2741	1 2751	1 2761	1 277	7 2781	1 2791	<b>:</b> -
_	GCCCTTGCGA	TGAGGCGGAG	GECETTOCGA TGAGGEGGAG CAGGAGATEG ACCAGGAGCA GTTGECEGTG CECGAAACEG TGECEGAACE GEGGGECAAG ACTECTAAGE GEGGAEAGT	ACCAGGAGCA	GTTGCCCGTG	CCCGANACCG	TGCCCGAACC	GCCGGCCAAG	ACTCCTAAGC	GCCGACCAGT
2801	281	282	2831	2841	1 2851	1 2861	1 2871	7 2881	1 2891	<u></u>
_	gaagaaggat aaggcagata aggcagataa ggacaagtog accagaggco caaagaaacc stsccttogo acgacaagga tgacgagcto aagacaacg	AAGGCAGATA	AGGCAGATAA	GGACAAGTCG	ACCAGAGGCG	CAJAGAJACC	Grecerress	ACGACAAGGA	TGACGAGCTC	AAGAGCAACG
2901	2911	2921	1 2931	2941	1 2951	1 2961	1 2971	1 2981	1 2991	
_	ACGTCGACAA C	CAACGAAGAG	ACGECGACAN CANCGAAGAG TECGGEGACA CAGAGGGEGG EGAGEGEEEG ANGECECAAG GACATEGACA ACGTGGAEGA AATGGAEGAE TEEGAEETEA	CAGACGGCGG	CGAGCGCCCG	AAGCCCCAGC	GACATCGACA	ACGTGGACGA	AATGGACGAC	TCCGACCTCA
3001	3011	3021	1 3031	3041	3051	1 3061	1 3071	1 3081	1 309	
	TGOTGGCGTT C	TCCACCATC	TOGOGGOTT CICCACCATC CICGCAGACT ICAAGGACCT LACCCAACGA GIGAAAGCIC ITICGICCGI GCICACGGAC GIGCAGGGG CCGGCATACG	TCAAGGACCT	TACCCAACGA	GTGAAAGCTC	Trresteer	GCTCACGGAC	GTGCAGGCGG	CCGCCATACG
3101	3111	3121	1 3131	3141	3151	1 3161	1,716		3181 3191	-
	CAGGAGCTTC TCGACGCTCG GCAAGGCTCT GACGGAGGCG GCCCACATCG CCAACACGG ATCTAAGCCA GTCACTGCGC CTCGCAAGAA GAAGGCCGCC	CGACGCTCG	GCAAGGCTCT (	SACGGAGGCG	GCCCACATCG	CCAACACCGG	ATCTAAGCCA	Greactecee	CTCGCAAGAA	GAAGGCCGCC
3201	3211	3221	1 3231	3241	1 3251	1 3261	1 3271	1 3281	1 3291	
10	GCCTGCAAA AGIAGGA CTAAATAGGA AGGTCGGTA TGCGGGCGCT GCACCTGTCA GACGGCAAAC TITITITIGA CAAGGAGGTG ACGCAGCCGG	TAGCCCA	CTAATAGCG A	GGCTCGGTA 1	F4R-	SCACCTGTCA G	BACGGCAAAC	rrrrrrca c	CAAGGAGCTG	ACGCAGCCGG
3301	3311	3321	1 333	334]	3351	3361	3371	3381	1666	
~~~	recedanda enacecede finederdree findedalgar eccanteea eccaneter eggandings egigineda enganetre Agretoego	AACCCCGGG 1	TACGCTGTCC 1	TGCGAAGAT	CCGGATCCCA	CCGCACCTCT (	CGGATGTGGT	CGTGTACGAG (	CAGGACCTCG	AGTOTGCGCA
3401	3411	3421	3431	3441	3451	3461	3471	3481	3491	
_•	ocadodeere Aretregres Goedecanede Candodeecon Andendraer Ternessacs Eggaenests Gagescere Cadeecors Cadeecors	retregree	GGCGCGACGC	MGGGCGA 1	AAGCAGTACT '	TCTACGGGCG (	GGACACGTG	CAGCGGCGCA C	מפככפתכפם כ	AACGCCGTG
3501	3511	3521	3531	3541	3551	3561	3571	3581	3591	
-"	megracos recreses carsarcas araanseer reansanda escentese fecasases assessand esansase sermente	SCACCGCGT	CATGAACAAG A	TANACGCCT 1	CATCGACGA (	CACCTCGCC 1	CCGGCAGCG	NGGCCGAGGC G	CAGATGGCC G	CCTTCCTGC

Fig 7.:

TCATGGAGAC GAGCTTCTTC ATCCGCGTCG GCAAGACGCG CTACGAGCGC GAGAGCGGCA CCGTGGGCAT GCTCACGCTG CGCAACAAGC ACCTCGCCGA 3901 3911 3921 3931 3941 3951 3961 3971 3981 3991 3991 AGANCTIGG CACCIACGG CACCIACG CACCIACGG CACCIACGG CACCIACGG CACCIACGG CA geccanagae agranganan receesrese erregisese haganeeas resessaces sittacesis escanagase hacaserer escassers COTCGGGTCT GGGACCCGGG COCGCCCGAC AGGCTGCTGT TCGACCGGCT CAGCGAGCGC CGCGTGTACA CCTTCATGCG ACGCTTCGGC ATCCGCGTCA CACCTCCGTG CGGCAGACGG CCGAGACGGT GGGGCACACG CCTCGATCT CGCGCAGGGC CTACATGGCC ACGGGGGTGC TCGAGCTCGT CAGGGACGGC crececerses cesceces careascese crecesces ressentes coresert ecocaecres ecocaecres ecocaaces democae 3721 3731 3741 3751 3761 3771 3781 3791 4011 4021 4031 4041 4051 4061 4071 4081 4091 4181 3841 3851 3861 3871 4151 4161 4171 4211 4221 4231 4241 4251 4261 4271 cecreasda oracrecase assecerres hosacrinea arreseesse assasegas deresessas es 3651 3641 4101 4111 4121 4131 4141 3831

Fig 7.

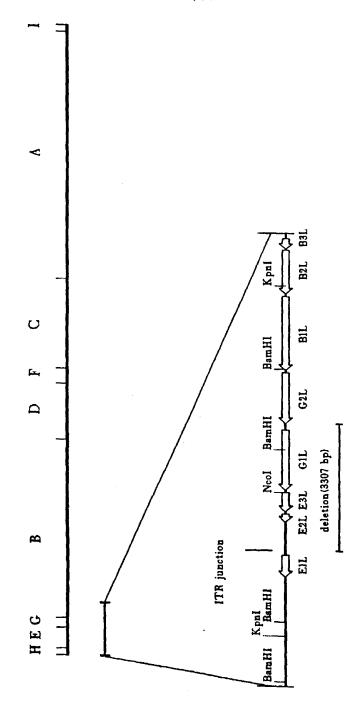


FIGURE 8. BamHI Map of Orf Virus NZ-2 showing the location and orientation of the reading frames for the putative genes E1L(ORF-3), E2L, E3L(ORF-PP), G1L, G2L, B1L, B2L, and B3L.

Figure 9. Nucleotide sequence of part of the BamHI E and BamHI G fragments from orf virus strain NZ-2 showing potential insertion sites.

		77		1	Ţ	51	19	7.	81	91
	AGTGAGCGG	CGGCCATA	adtorandes de descentar anatarte <u>na I</u> tenetente de de cencetars de	Trgactgatt	cecrestase	CGAGCGAAGG	GCGGCGGACA	AGGGCGCGGG	ATGCTGGTCT	AATCTACTAA
ĭ	101	111	121 131		141 151		161 17	171 11	181 1	191
	GGCCGATTAC	2 AMANACGGAS	GOCCONTINC NAMACGGAT GOGAGACCOS GAGGGAGAGS GTCACAGCTC CGAGCGGTGC ATCCGCGCCA GCTGGCGGCG CCACTCGGCC GCGGGCCGCG	GAGGGAGAGG	GTCACAGCTC	CGAGCGGTGC	ATCCGCGCCA	GCTGGCGGCG	CCACTCGGCC	ಕ್ಷಾರ್ಥವಾಗಿ ಕ್ಷಾರ್ಥವಾಗಿ
201		21, 2,	221 231	11 241	11 251	1 261	11 271	•	281 2	291
	   ೧೯೮೮ ೧೭೩ ೧೮	, ccecerrera	ecceccasa coecamen dececcese aceseasa asseasas concases danences ecaseeses deacenem dansisase direcasen	ACCGCGACGC	AGGTCAGCTC	GAACTCCGGC	CCGAGCGCGC	GCACGTCGTA	GATGTGCACG	GTCGCGACGT
301		31, 35	321 331	1 341	136 11	1 361	371		381 33	391
	TCAGCAGCAG	, ೧೯೧೮ ೧೮ ೧೯	דכאסכאפראם לפבסכרכידים לפכאמכנמאם לפאאמסיכסב לדסכאיזמכנם לכפסנפאמנם לפדאנאכנמם לפאפאמניניב לכמכנמכנסד לכמכמאכנאנ	CGAAGGTCGC	GTGCATGCCG	GCGGCGAGCG	GGTACACCGG	CGAGAGCGTC	CCGCCGCCGT	GCGCGACCAC
401		411 42	421 431	1 41	1 451	1 463	(1)	1 481		161
	CGCCATGTGC	. cecceareac	כפככאזפזיםי בפככנסורסי בפככסנאבסים באססכאסמים אככסאססכם אסכנסודכסי פכסנאכססס בככמכנדכנא במאסספכסם בסנכאפרסככ	CAGGCAGGTC	ACCGAGGCGG	AGCCGTTCGC	GCGGACGGG	CCCCCTCCA	CGAGGGCGGC	CGGCAGCGC
501	i sti	13 221	1 831	1 541	1 551	195	1 571	•	581 - E1L 591	Ξ-
	   GGCGCCGCGG	GGCGGAAGAG	GECECCECES GECEGRAGAS CONSCIONAS AGGAAGENCA GESCOACEAS CONSCISCE CEGASEASES TGCGEGGERA GESCISCATS ÉTISTICSET	AGGAAGCCCA	GCGCCACCAG	CGCGAGCGCG	CCGAGCAGCC	TGCGCGGCGA	 GGGGTGCATG	crrerreser
603	9 611	11 621	11 631	1 641	1 651	1 661	1 671	1 681	1 691	1
	acconomica	corcreeced	GCGFTTGG CGTCTGGCGG GTGGAAGGCG GGTGTGGGGT GTCCGCGGCT GCGGCGGGAG GGTCTCGACT GCTAGGCGGT CCTTTTTCAC TTTGCTCCGT	GGTGTGGGGT	GTCCGCGGCT	GCGGCGGGAG	GGTCTCGACT	GTCTCGACT GCTAGGCGGT CC	CCTTTTTCAC	TITITICAC TITGCICCET
701	ITR	711	721 7	731 7	741 7	751 7	761 7	7.1	781	161
	GGCGCCTGGT	CCGGGGCAAG	$\frac{1}{2}$ დაცილის გადის გადა გადის	) <u>599333</u> 5556	)    -	SCTACCCCGC (	)	פכפככפככא	2292822828	CAGCGGCGC
801	811	1 821	j 83j	841	188	198	871	88.1	168	: : : : :
-	CACCGCGGGC	0000000000	ີ ວ	GCCGCGAGC	0000000000	ಶಾಂತಿರಾಧಿಕ್ಕಾರ	CGCGCCGAGG	ವಿವಿವಿಶಿಶಿವಶವಿವ	ರವಿಶವಿಶವಿಧಿಶವಿಶ	AGCAGCAGCG
106	911	921	931	941	156	196	971	981	991	
_	GCAGCCGCGC	GTCCAGCGGG	פראשרכשרשר שדיכראשרששם ברשרכשרשם שנאסלשרשם מנפראפראשר שרפרנאשנם מהאסרכשר בשרפרפרני בשנכנפרפרני שאסרנפרנים למכפרפרני	эслососсес	GCGCAGCAGC C	3ccaccaaca	SCAGCCGCCC (	GCCGCGTCC	9000000000	ಎಂತಿಂತಿಂತಿಂತಿಂತ
1001	1011	1021	1031	1041	1051	1061	101	1081	1091	
	CGCCGCCAGC	AGCGCGCGCA	coececenes nocesceses ecnecesses denocesses ecoecesens chaorestes hearches responent cantreres dinatracas	3GAGGGCGCC	GCGCGCGGAC C	SAGGTGCTCC )	ACGAGCAGGG 1	regreaceaa c	GATTCTCGA	SAACTAGGAG

Fig 9.1

	1211	1131	1141	1151	1161	11711	1181	1191	
ខ្ល	ACAAGGAG	AGACGTTATA	TCATOTOTOA COACAAGAA AAAGOTIATA TIAGGOGGO CCTACITCAC TITGAAGATO OTGTAAAGTO TIAAAACTIG AACACCGTIC ACTCCACCAC	CCTACTTCAC	TTTGAAGATG	GTGTAAAGTG	TTAAAACTTG	AACACCGTTC	ACTCCACCAC
1211	1221	1231	1241	1251	1261	1271	1 1281	1 1291	
_ <b>5</b> ,	GPCCTGCC	CCAMAGCAA	TOCCGTTACC GTOTCCTGCC CCAAAAGCAA CCACAGTGCT TTTTCCACCA CCTGTTCCAA ATCCGTTCCA AAAGCTCCCA TCCATTGTTG TTAGAACTTT	TTTTCCACCA	CCTGTTCCAA	ATCCGTTCCA	AAAGCTCCCA	TCCATTGTTG	TTAGAACTTT
1311	1321	1331	1 1341	1351	1361	1371	•	1381 1391	
۲	TAGGTTGE	TTAGTTCCAC	CAGAIGITIC ICTAGGITGI ITAGIICCAC IGCAAGITIC GACCATIAIC GITACIGGAC AIGCIGITGG IAATGAGIIT AAIAACCAAI CAIAAAAIA	GACCATTATC	GITACIGGAC	Argererree	TAATGAGTTT	AATAACCAAT	CATAAAAATA
1411	1421	1 1431	1 144]	1451	1461	1471	1481	1 1491	
k	TATANAGC	TAATAAAGTA	GITALANITI GITALANAG TAALANAGIA GCAAAGAC <u>II. I</u> AATGITATA TITIGCCIAA ČCCICCGILA ACACCACCAL IAACACCACC ACITAAGCII	AATGTTATA T	TITGCCTAA C	CONCEGURA	ACACCACCAT	TAACACCACC	ACTTAAGCTT
į	1521		931 1541 1551 1561 1571 1581 1591	1551	1561	1571	1851	1591	-
ੋਹ -	CTACCACC	TCCAACACAC	THACHACAR CACHACACA TOCAACACA ATTOTTTOT CTANAGGICC CCAAATTOCA CCICCIGAAC TIGGACGIII TACAGCACT CCGGGIGIAC	CTAAAGGTCC C	CANATICCA	crccrease ;	TTGGACGTTT	TACAGCACCT	CCGGGTGTAC
•	1621	1631	1641	1691	- E2L	1671	1681	1 169	
E	TAGAAGIT	CCACTGTGAC	THOCOTACCE THINGANGTE COACTOTONE IGENOATATE ATACTOTICE TELECOAGGEN TGATTANAGE GIGTTGIANT TAGTOTIATE TAGGCAACTG	 ATACIGICCI	CTCCAGGCA 1	GATTAAAGT	STGTTGTAAT	TAGTGTTATC	PACGCAACTG
	1721	1731	1771 1751 1751 1771 1771 1771 1771 1771	1751	11111111111	1771	1911	1971	1
I D	GAATAAA A	IGAAG <u>CTA</u> CA 1	TGGGAGACKC TGGAATAAAA AGAA <u>GIla</u> ca Titiacaatt Iigahiagci Gaigiaccac Gctgiaicge Ggccaccaca Agcacccat Ccagiagaac	PESE   TGATTAGCT G	ATGTACCAC O	crerarcec o	GCCACCACA	AGCACCCGAT C	CAGTAGAAC
i	1821	Ì	1891 1861 1861 1861 1861 1861 1861 1861	1821	1981	1971	1881	1881	-
- ₹:	99090090	TCAGTGTTGT	CAMATICCAGA GICGCOGOG TCAGIGITOT CCAMGCAGIT ANCCICTIGA ACIGCIGGG ACGAIAIGGG TICGCAIAIT AGCIGAGCIA ICCIGICICC	ACCTOTTGA A	crecreece »	CGATATGCG T	TCGCATATT	AGCTGAGCTA 1	cererece
-	1921	1931	191 191 193 194 1951 196 1971 1981 1991 1991 1991 1991 1991 1991	1951	1961	1971	1981	1661	
9 :	AAAGTCAC 7	TGTTTCCAAA (	CTICTIANCC TCANAGICAC TGTTTCCANA GTTAAGAGC ACCACTCCGA CGTTGCCTCG GTAGTCTTGG TCGATCACGC CAGGGCCCAC GTCGATAAAG 2011 2021 2021 2031 2041 2051 2051 2051 2051 2051 2071 2071 2081	CCACTCCGA C	Grrecice 6	TAGICTICG I	CGATCACGC C	AGCGCCCAC 0	TCGATAAAG
≪ ∵	AGGCCAGA A	ACGTGGTGCT 1	TOTTTOACTG CAAGGCCAGA ACGTGGTGCT ATGCGTCCGT ACGACCAGA AGGGGCTTT ATCAGAAGGT CAGTAAATAC TACGGGACTG CAATGCGAAG	GCAACCAGA A	  GGGGCTTT A 	TCAGAAGGT C	AGTAAATAC T	ACGCGACTG C	AATGCGAAG
F -	CGTATGCA C	TACATAGGT C	GGATGACACA GTCGTATGCA CTACATAGGT CTAAATCCTGC GCCACCAGGA GATCCTCTGG CTGGTATAGT GGCGTTTTGG CTGAGGCGAA CAACCTGAAG	GCACCAGGA G	Arcererge C	TGGTATAGT G	GCGTTTTGG C	TGAGGCGAA C	AACCTGAAG
	<b>\</b>	→E3L 2231 Ncol	2241	2251 2261 2271 228	2261	2271	2281 ***** PE3L	2291	
ខ្លួះ	AGAACT CC	GTTTCCGTG TGCCAGACT <u>CCATGC</u> CTAG GGT	agitilogia tagcagaaci <u>ccaigg</u> ctag gaiggcgagc geccgaicga ctacggestg tacaaittac actiticca gaaaa <u>ica</u> g gggcgggca ##############################	CCGATCGA CT	ACGGGGTG TA	CAATTTAC AC	TTTCTCCA G	ACTITCTCCA GAAAAAICAG GGGCGGGTCA	660664CA

Fig 9.

	TGAGGCCGGC 111111111111111111111111111111	CAAAAAGGCG 	GCGATCTCCT	CGCACAGCAG	CACGGCCGCC	AGCCGGCAGC	AGTCCACGAC	AGCGTGCGC	GCAGCATCA	GCGCTCGAG	90000000	recorce
1 2391	GCATGEGGG GGGLAGTCC AGCAGGGAGT CGTACGACA GAAGCACAGG ATGGAGGTCA CGATGTCCGG CGCAGGGG CACGGGCA. TGAGGCCGGC	GATCTGCTCG GCCAGCGAGA CGCGCAGCCG CATCATGCAG ATCTTGCCGA AGAGCGCCGT CCCGTAGATG GGGAACTCGG CCGCGCGCTC CAAAAAGGCG FEEFFEFFFFFFFFFFFFFFFFFFFFFFFFFFFFF	TTTCTGCACGA AGAGGGCTTT CGCGTGCTCC GAGGGGCGCA GCACGTCCAA CAGCGTCGCG TCCGTGTGGC AGCGCACGGC GCGCATGCTT GCGATCTCCT	SCHOGGAGGG GGGGATGACG GTCGGCTAGT CCGCCAGCGC GCGCTCCGCC AGCATGCGC GCCCTCGCC GCGCAGCGC AGCTCCTGCA CGCACCAGCAGCAG  271, 272, 272, 272, 272, 274, 274, 275, 276, 276, 277, 277, 278, 278, 279, 279, 279, 279, 279, 279, 279, 279	GOGGECTEC GAGEGTEGGA ACACGTGGGC CCATTGETCA GATGTGATCA GGGGGGGGG GAGCAGCTEC GTCGGGGGC GGGGGGGGA CACGGCGCCCCCCCC  1311111111111111111111111	Freededeck correctede descreeke recharace cacaragee caregoegae Argreeres derecedee caregoeree Ageogoage French Franch Fra	Nagegeogra derghaeare decoegegear dengegegar ergehagare fremregea dorrengare engecegee fedagenean Agreeaceae Hillight Hillight Hi	TAGGCCCGT 6	3291	GGTTCGCGC C	GTTCACGCG C	POCAGCAGGA GOGGGGGAC CTCGAGGTGG GGGGGTGGG GGCGGGAGAA AGCGAGGAAG GAGGAGAGGA
1 2381	CGCAGGGG C	GGGAACTCGG C	AGCGCACCGC (1111111111111111111111111111111111	GCGCAGCGCC A	GTCGGCGGCC G	GCTCCGCGC C	CAGCCCGCGC 7	GGGTCCACGC (	COCACGCGTC C	PCAGGGGTTT G	rerrecer c	GCTGTCGCA G
1 2371	CGATCTCCGG C	CCCGTAGATG C	TCCGTGTGGC 7	caccercace   d	GAGCAGCTCC G	ATGTGCTCGA G	GOTTCAGGTC C	TACGGCGGCC G	SCGTCCACGA C	correctors corrected in the correction in the co	   ACGCCGTAG G   1111111111111111111111111111111111	AGGAGAGCA C
1 2361	ATGGAGGTCA C	AGAGCGCCGT (111111111111111111111111111111111111	CAGCGTCGCG 7	AGCATGCGCG C	GCGCGCGCGC   111111111111111111111111111	CGAGGCCGAC 1	TTGTTGCGCA G	CACACGCGTC 1	GCGCCGTAC	SGCGTCANGC C	CAGCGGCGT 1	GCCAGGAAG G
2351	GAAGCACAGG /	Arcridecea A	GCACGTCCAA C		GATGTGATCA G	CGCATAGGCC C	CTGCAGGTTG 1	Argergrace of	scaggerrer o	gccgccAgc q	CGTACACGT C	GCCGCAGAA A
2341	COTACGACAG	CATCATGCAG	GACGCGCGCA (	CCGCCAGCGC (1111111111111111111111111111111111	CCATTGCTCG	TCCGAAACCG	3041	3141	######################################	GCACAGCAG C	TGGTTCTTG G	COCCOTCOC
2331	AGCAGCGAGT	CGCGCAGCCG	CGCGTCGTCC 2631	Greacerage of 11111111111111111111111111111111111	ACACGTGGCC	GCGCAGCATC 7	GCCGCGCGGT (	AGGTCGCCAT	3231	1666 1333333333333333333333333333333333	GCGGATCCA C	TCGAGCTCG G
1 232	acachaorec	GCCAGCGAGA 11111111111111111111111111111	AGAGCGCCTT	GCGGATCACG	GAGCGTCGGA 2821	CGTTGTTGCG - 2921	GCTGAACACC	CAGCTCCCGT J	GGAGATCIT	COCGGCGGAG A	GCACGTCCT C	CCGCGCGAC
1 2311					CGGGGCCTCC GAGCGTGGGA ACACGTGGCC CCATTGCTCG GATGTGATCA GGGGGGGG GAGCACCTCC GTCGGGGGC GGGGGGGGG CACGGCGCGCCCCCC  11111111111111111111111	GTCGCGCGCA COTTOTTGCG GCGCACCATC TCCGAAACCG CGCATAGGCC CGAGGCGGA ATGTGCTCGA GCTCCGCGCC CATGCGCACC AGCCGCAGC 3.111111111111111111111111111111111111	AGGCGCCOTO GCTGAACACC GCCGCGCGGT GCAACGCGGT CTGCAGGTG TTGTTGCGCA GGTTCAGGTC CAGCCCGCG TCGAGCACGA AGTCCACGAC 	GCCGCGCTCG CAGCTCCCGT Aggregocar grantgeage Argeretree Cacacgcgre facgccgcc gggrecac ctaggcccgr dagccrer dagccrec facgrecoc	ACCATGCCCT CGGAGATCTT GGCCGTGCC GCGAGGTGGT GCGCCGTAC GCGCCCACGA CGCACCACGA CGCGCGTC CGCGCCGGG CGCAGCATCA 1111111111111111111111111111111111	TETCCACGAA CGCGGCGAA ACGCGGCGG AGACAGAGA CGCCGCCAGC GGGGTCAAGC CGTTGCAAGC GCAGGCGTTT GGGTTCGCGC CGCGCTCAG 	CAGCAGCGG AGCACGTCT CGCGGATCCA CTGGTTGTTG GCGTACACGT GCAGCGGCGT TACGCCGTAG GTGTTGCCCT CGTTCACGCG CGCGCCCGCG	TCCAGCAGCA GCCGCGCAC CTCGAGCTCG GCGCGTCGG GGCCGCAGAA AGCCAGGAAG GAGGAGAGCA CGCTGTCGCA GACAACGACG CTGGCGTCGC
2301	2401	2501	2601	2701	2801	1062	3001	3101	3203	3301	3401	_F `

Fig 9.

3571 3581 3591	AGACCACGTO CGCGCCCGCO TCCAGCATA GGGGAACLAC CTCCGGCGC ACGCGACGTA ACTGCACGTA GGCGTGCAGC GGCGGGGGC CGCAGGACTC PHILITIAN HILLIAN H	CTTGGCTTTC ACGTCCGGCAC CGGCCTCCAA CAGACTCCCG CGCACTGCTC GTGCCGCGGG AAGTGCACGC AAAGGTGCAG CGGCGTGCGC	CCGTGCTCC CGCGGAAGTT CACGTCTGCG TCGGTGGCTA CAAGGGGGGG ACCGTTTCG AGGTCCACCT GCCCGAACTC CAGGTAGCGG AAGAGCAGGT	3811 3821 3831 -GIL 3841 3851 3861 3861 3871 3881 3891 CGGGGTGCG GACCACGAG GACCACGAG GACCACGAG GACCACGAG GACCACGAG GACCACGAG AGAGCAGG GACCAATAA	391j 392j 393j 394j 395j 396j 397j 398j 398j	deotananot anamanctic tacamanac otacamango tacamagot amamagoco descesseac essetsesset ectessaset saatisseet		
1361	Acdccorcor J	CGCACTGCTC G	ACCGTTTCG A	3831 -GlL 3841 3851 3861	3961	MANANGOCG G	} ! ! ! ! ! !	
1 3551	CTCCGGCCGC	ACGATCTCCG	CGAGCGCGCG	3851	3951	FACANAGGT A	6 4 5 1 1 1 1	
3541	GCGCGACCAC	CAGCACGCGC	TCGGTGGCTA	-Gll 3841	394]	TACALANGG 1	1 3 0 0 0 1 1 1 1 1 1	
3531	TCCAGCATGA	CGGCCTCCAG	CACGTCTGCG	3831	3931	PACAMANAGE (	1 1 1 1 1 1 1 1	An John
3521	3621	ACGTCCGCAC 111111111111111111111111111111111	CGCGGAAGTT (	3821   2ACCACGACG	3921	MANAACTIC 1	0 0 0 0 1 1 1 1 1	andhother of deletion
3511	AGACCACGTC   11111111111111111111111111111111111		 	3811	391	recrandance A	4011	-
3501	3601	3701		3801	1901	_ "	4001	-

Fig 9.4

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FIGURE 10. Orf Virus Transcriptional Promoters.

## Early Promoters

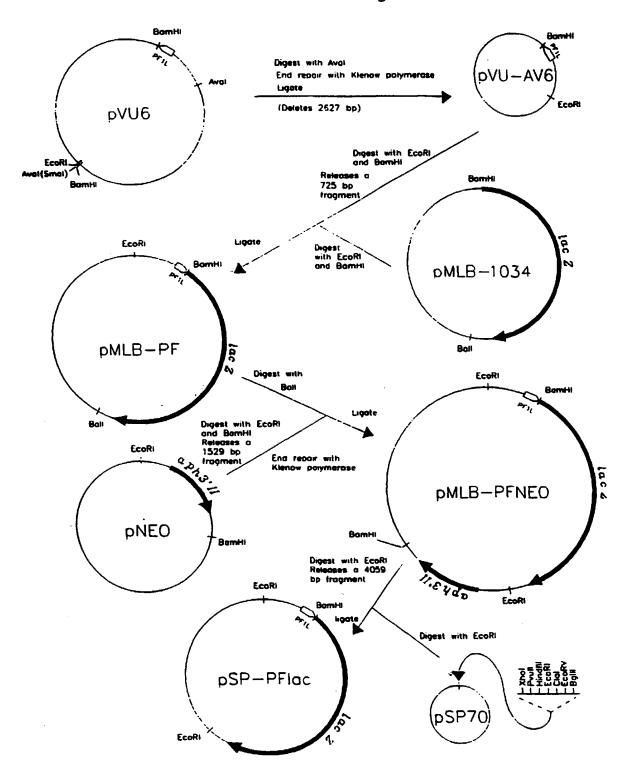
E3L (OR	(F-PP)	GAAAGTGTAAATTGTACACCCCGTAGTCGATCGG
E2L (OF	RF-1)	AAAATTGTAAAATGTAGCTTCTTTTTATTCGAGA
E1L (OF	RF3)	GCAAAGTGAAAAAGGACCGCCTAGCAGTCGAGAC
GlL		GATGAGTGAAAAAAGATTTCAATATTTGTAAACG
G2L		AATAACTGATAAAATATGTTTTTTTGGTTTTGGT
BlL		ATAAATTAAAATTAAAGCGCGGAGGCTCGAACGC
B3L		AATTATTGAAAATGTAGGCGCGATAAACACACGT

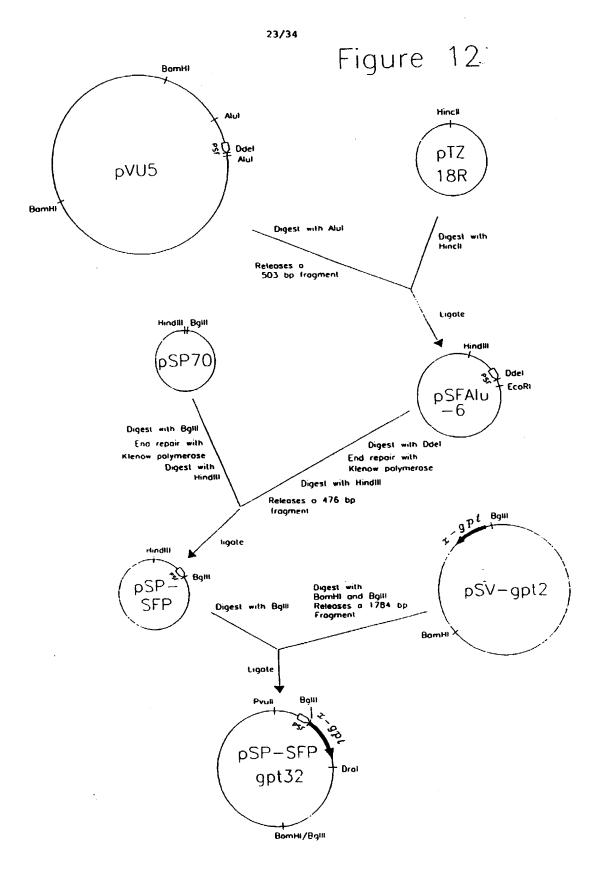
### Late Promoters

F1L	GCCGCCATAAAAGAGTTGTATATGATTAATTTTAATAAC <u>TAAAT</u> GGATC
F2L	TTTCAGTTTTGAGACGGTCTCGCGCGCCCTTGCCGTCCT <u>TAAAT</u> GGATT
F3R	ACTCTTAAAAAATCGGTATTGAAAGTACGCACCACCAAA <u>TAAAG</u> CGTCG
F4R	CGCAAGAAGAAGCCGCCGCCTGCAAGAAGTAGGCGCACTAAATAGCGA
B2L	AAGACTTTCCCTGAAGCCCTATTATTTTTGTGAGATAAATAA
HE2L	GGAGCTGCGCGAGCTCCGCGCCAACGAATAATTCTGCACA <u>TAAAAGAT</u> G
HI2L	ATATTAGATAACCGCTGTGTTGCCGTCTGTAATTATTTAATTATAAATG
HI1L	GTAATAAGGATTAACATACGAAGTTGGGATAATTATTTAATTATAAATG
C1R	TTCGTGGACATCGTCGTGGACTATGTAAATAACTCTGAGCAGG <u>TAAA</u> TG

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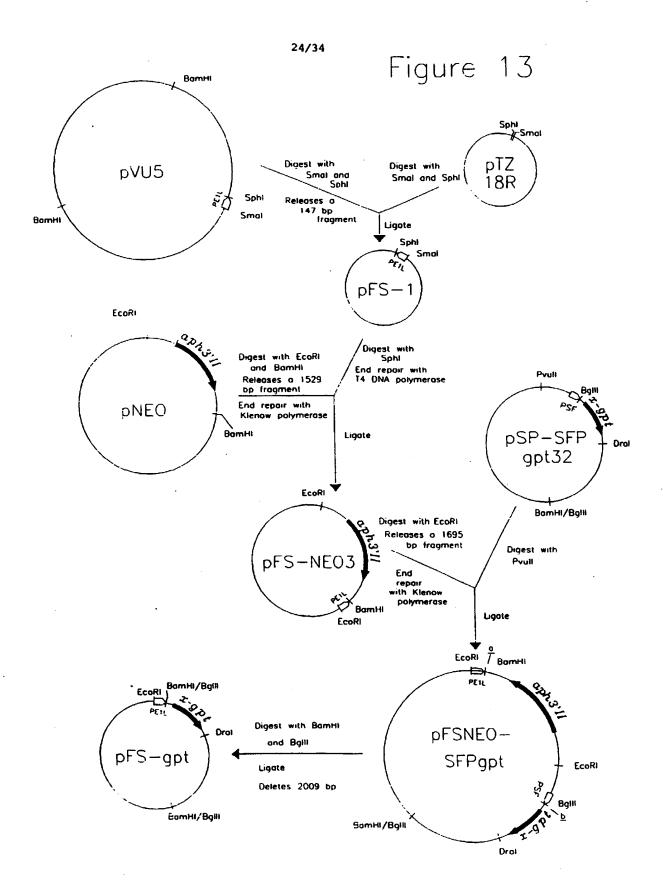
# Figure 11



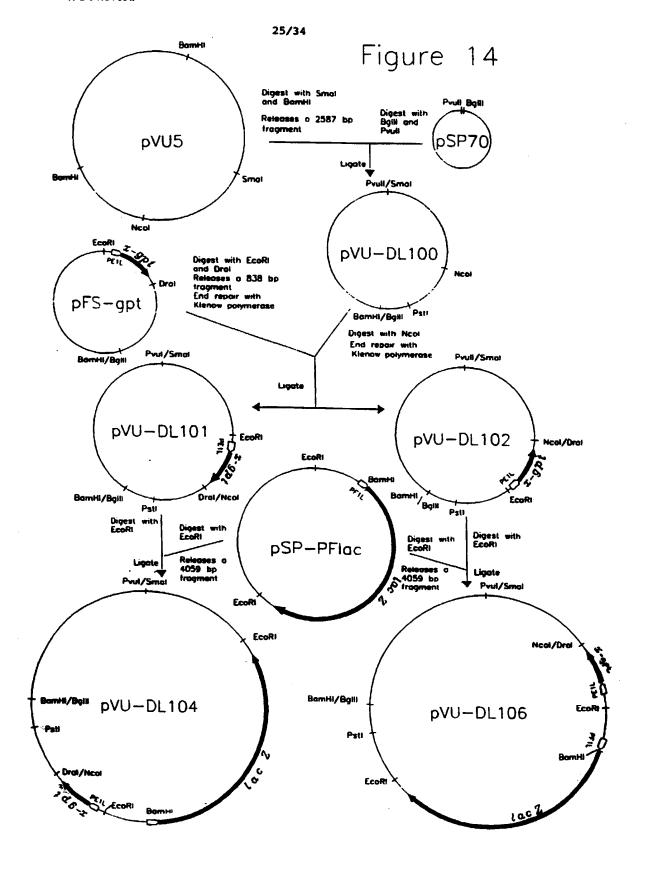


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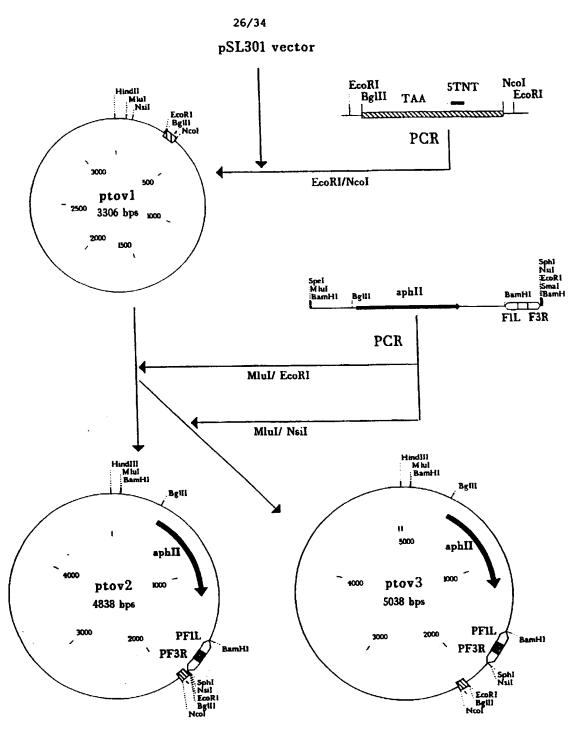


Figure 15. PCR amplification steps involved in the construction of ptov2 and ptov3.

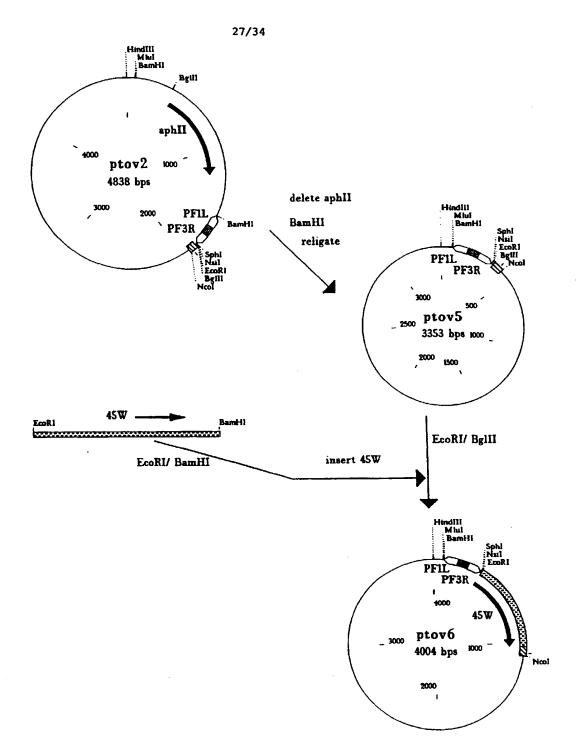


Figure 16. Cloning of the T. ovis 45W antigen into ptov2.

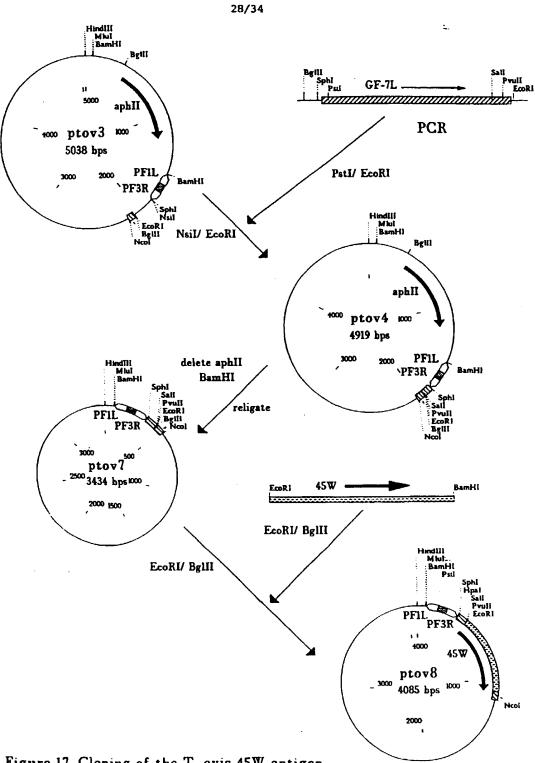


Figure 17. Cloning of the T. ovis 45W antigen into ptov3.

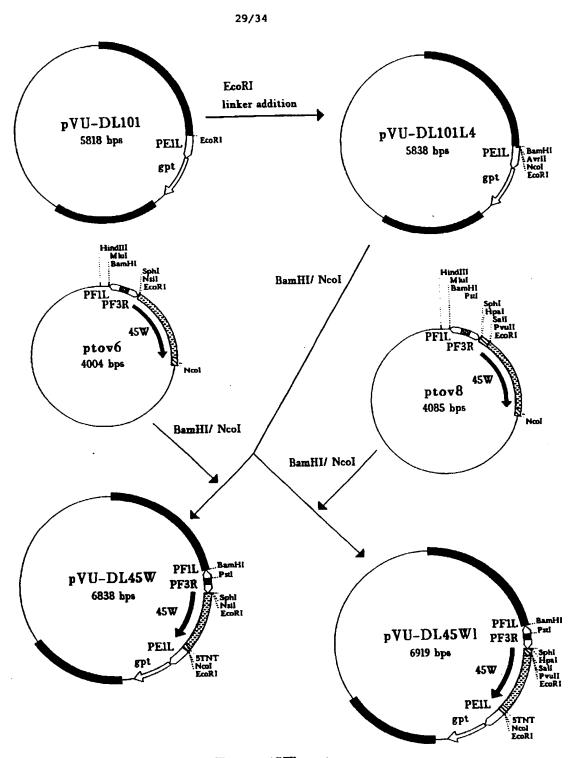
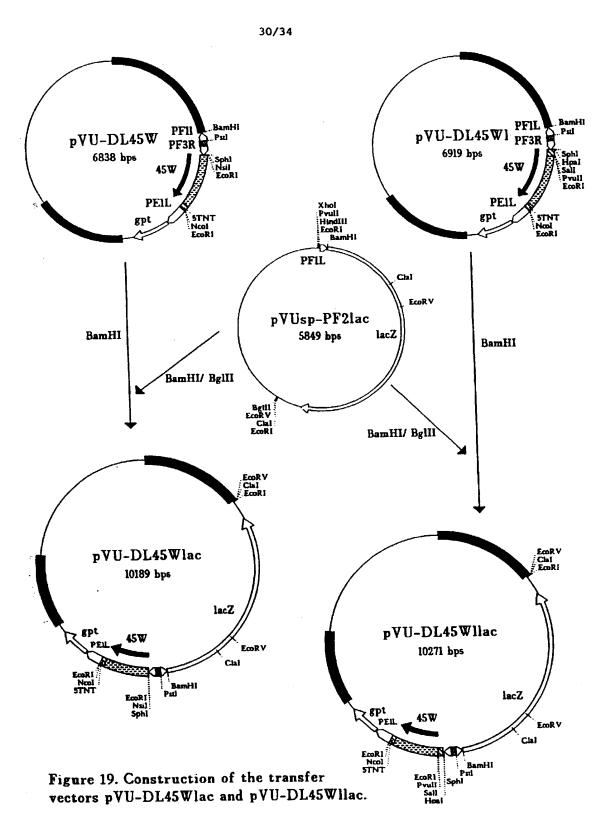


Figure 18. Insertion of the T. ovis 45W antigen into pVU-DL101.



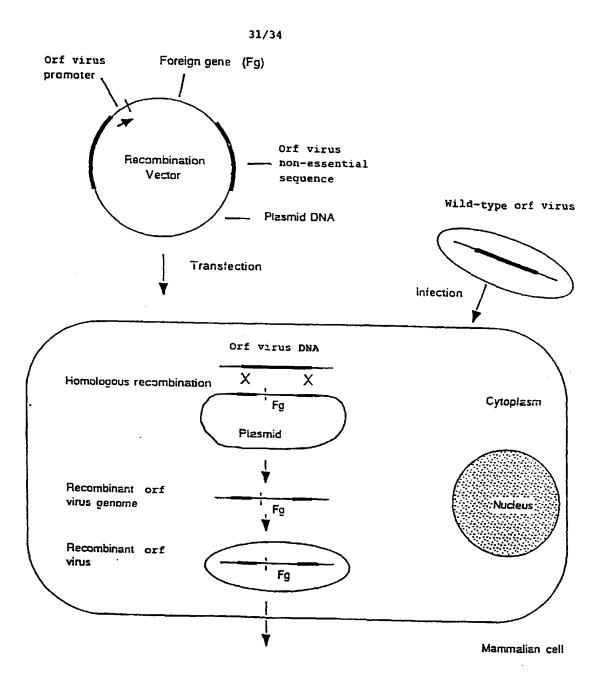


Figure 20. A strategy for the generation of recombinant orf virus.

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zxs-1 GAT CCC G<u>CT CGA G</u>AA CTT CAA *Xhol* 

zxs-2 GTC <u>AGA TCT ATG CAT</u> AA<u>A AAT TT</u>C GCA TCA GTC GAG ATA

\*\*BglII\*\* Nsil\*\* Apol

zxs-3 GAC ATG CAT CAG TGC CAT GGA ATT CTC GCG ACT TTC TAG C

Nsil Ncol

EcoRI

zxs-4 GAC <u>GGA TCC</u> GTA TAA TGG AAA GAT TC

BamHI

Figure 21A. Primers used for the amplification of orf virus sequences used to create the transfer vectors pTvec1 and pTvec50.

1 11 21 31 41
GACTGATGCG AAACGCGCGG CGGCGCCGCG ACTTAGCTTA TCTCGACTGA
\*\* \*\*\*\*\*\*\*\*\*
zxs-2 primer

51 61 71 81 91
TGCGAAATTT TTATGCATCA GTGCCATGGA ATTCTCGCGA CTTTCTAGCT
\*\*\*\*\*\*

Apol Nsil Ncol zxs-3 primer

EcoRI

101 TCTCAGACTG ATGCTAC

Figure 21B. Sequence of modified intergenic region between the RNA polymerase subunit gene, rpo 132, and (H)IIL in pTvec50, showing new created restriction sites for the restriction enzymes *Apol, Nsil, Ncol* and *EcoRI*. The priming sites on the original OV sequence for the zxs-2 primer and zxs-3 primer are markerd by asterisks; the newly created transcriptional termination signal (TTTTTAT) is shown in bold type.

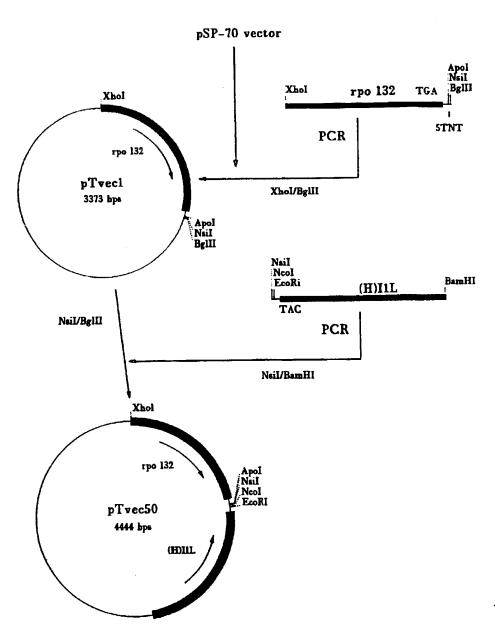
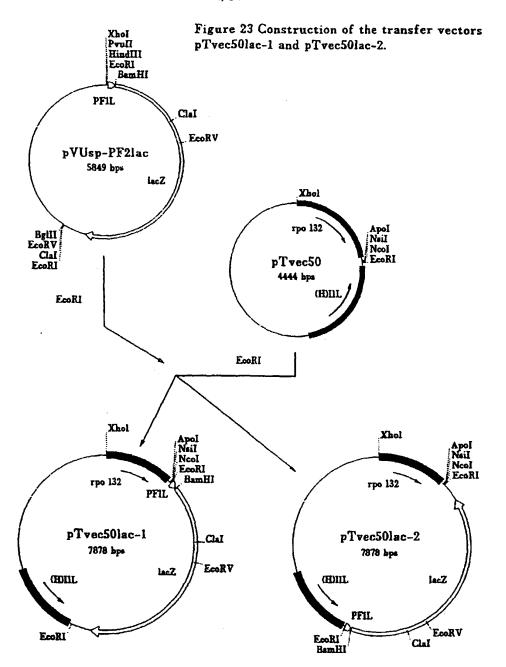


Figure 22 PCR amplification steps involved in the construction of pTvec-1 and pTvec-50.

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## INTERNATIONAL SEARCH REPORT

International Application No.

		I CIM	2 9 //00040
A.	CLASSIFICATION OF SUBJECT MATTER		
Int Cl <sup>6</sup> : Cl	2N 15/86, 5/10; A61K 39/275		
A a a = = = = = = = = = = = = = = = = =	International Detart Classification (IDC) and but	national electification and IBC	
	International Patent Classification (IPC) or to both	i national classification and IPC	-
В.	FIELDS SEARCHED		
Minimum docu IPC6	mentation searched (classification system followed by o	classification symbols)	
	searched other than minimum documentation to the ex BASES BELOW	tent that such documents are included in	the fields searched
WPAT - Par	base consulted during the international search (name or apox or orfvirus: or parapoxvirus: or orf () virus  Parapoxvirus or orf () virus		
C.	DOCUMENTS CONSIDERED TO BE RELEVANT	Γ	
Category*	Citation of document, with indication, where ap	propriate. of the relevant passages	Relevant to claim No.
х	RECOMBINANT POXVIRUSES (1992) Chapte "Parapoxviruses: their biology and potentional a Robinson AJ and Lyttle DJ eds M. Binns and G. In particular pages 310-316	as recombinant vaccines" by	1-25
Y	JOURNAL OF GENERAL VIROLOGY (1995) Fleming SB et al. "Genomic analysis of a transp reveals a 3.3 kbp region of non-essential DNA" See entire document.		1-25
x	Further documents are listed in the continuation of Box C	See patent family annex	
"A" document or control of the contr	al categories of cited documents:  "The ment defining the general state of the art which is presidered to be of particular relevance or document but published on or after the national filing date ment which may throw doubts on priority claim(s) ich is cited to establish the publication date of er citation or other special reason (as specified) ment referring to an oral disclosure, use, ition or other means ment published prior to the international filing but later than the priority date claimed unal completion of the international search	priority date and not in conflict with understand the principle or theory us document of particular relevance; the be considered novel or cannot be considered novel or cannot be considered to inventive at document is document of particular relevance; the be considered to involve an inventive combined with one or more other su combination being obvious to a pers	the application but cited to inderlying the invention he claimed invention cannot insidered to involve an istaken alone he claimed invention cannot we step when the document is ich documents, such son skilled in the art int family
	ling address of the ISA/AU NINDUSTRIAL PROPERTY ORGANISATION  1 2606	Authorized officer  J.H. CHAN	

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International Application No.
PCT/NZ 97/00040

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ategory.*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VTROLOGY (1987) vol. 157 pages 13-23 by Robinson AJ et al. "Conservation and variation in orf virus genomes"  See entire document, particularly last paragraph.	1-25
•		